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MYCOSIS OF THE BOVINE FETAL MEMBRANES DUE TO A MOULD OF THE GENUS MUCOR.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, December 6, 1919.)

An accurate knowledge of the number and variety of living organisms which may invade the uterochorionic space during pregnancy in bovines and multiply enough to set up localized or general disease of the chorion can only be gained by a cumulative study of the pregnant uterus before discharge of the fetus. After expulsion of the latter, adhesion of the placenta, the loss of pathological fluids held between uterine wall and chorion, the soiling of the placenta by the bedding, or its destruction by the cow, all stand in the way of an elucidation of those still unknown agencies of disease which may be of far more significance than is credited to them. The relation of *Vibrio fetus* to abortion may be considered as established. Occasionally *Bacillus pyogenes* is present in such numbers in the organs of the discharged fetus that tentatively it may be regarded as a primary agent in certain cases.[1]

Among a considerable number of pregnant uteruses obtained from abattoirs, the writer found one which deserves a brief description. The uterus was brought intact to the laboratory and the following facts were ascertained.

Case 182.—When the wall of the uterus was cut through there was found between uterine mucosa and chorion (uterochorionic space) a considerable amount of a turbid fluid full of small flakes. Over a region of the chorion about 20 cm. in diameter, corresponding with or resting on the left shoulder and thorax of the fetus, the cotyledons, five in number, are separated from the uterine wall so that the latter

[1] Smith, T., *J. Exp. Med.*, 1919, xxx, 325. Zwick and Zeller, *Arb. k. Gsndhtsamte.*, 1913, xliii, 1.

can be lifted away from the fetal membranes over this area. The pedicles of these cotyledons (maternal caruncles) are short, blunt projections of the uterine wall, varying in size, in color grayish with some hemorrhagic spots. The affected cotyledons are enlarged, the margins much thickened, and rolling cup-shaped over into the central necrotic portion. The chorion between these detached cotyledons is beset with yellowish gray, slightly elevated plaques, 2 to 4 mm. in diameter and a fraction of a mm. thick, producing a coarse goose-skin appearance. The subchorionic edematous tissue appears as a glassy, gelatinous layer 1 to 2 cm. thick. In one horn the chorion, over an area 6 cm. square, is beset with grayish to yellowish plaques containing minute hemorrhages. The fluid in the allantois at both horns is clear. The amniotic fluid contains a fairly abundant amount of fecal matter. The cervix of the uterus is normal and the external os is tightly closed and provided with the normal mucous plug.

The fetus is 25 inches long and normal, with the following exceptions. There is edema of the sheath of the umbilical vein within the abdomen, also slight edema of the capsule of the kidneys, and of the interlobular tissue of the lungs. The rumen contains considerable yellowish (fecal) matter, but the fourth stomach contents are still clear and colorless.

Although the gross appearances did not correspond closely to those found in the presence of *Bacillus abortus*, the case was considered one of infectious abortion due to this bacterium, until examination of the scrapings of the cotyledons showed a branched mycelium and no bacteria of any description. Cultures on slanted agar, sealed with sealing-wax and unsealed, of material from the surface of the chorion and from the amniotic fluid developed a mould. Cultures from the lungs and meconium of the fetus also contained only a mould, while those from the fourth stomach, spleen, liver, and one kidney remained sterile. Subsequent study of the moulds from placental and fetal cultures showed them to be identical and belonging to the genus *Mucor*.

The absence of *Bacillus abortus* from the cultures was confirmed by the results of inoculation of guinea pigs with the fluid exudate in the uterochorionic space, with amniotic fluid, and with contents of the fourth stomach of the fetus. The guinea pigs killed after 26 days

were normal and cultures remained sterile. Films from the chorion and the fluid exudate showed no bacteria of any kind. In the necrotic masses scraped from the cotyledons, a branched, non-septate mycelium was detected.

Tissues fixed in Zenker's fluid from various diseased and normal regions of the placenta were studied in sections in order to determine the relation of the mould to the tissues and the character of the tissue reaction. Although a variety of dyes was used, the mycelium stained very poorly and only careful focusing with a partly closed condenser made it possible to identify the filaments and trace them as far as the section permitted.

The remnants of the caruncles projecting from the uterine wall were densely infiltrated and covered with masses of polynuclear leucocytes. Among these masses the branched mycelium could be traced, penetrating them in various directions. The corresponding cotyledons with the sloughed off portions of the caruncles still embedded in them presented a variety of changes, consisting chiefly of necrosis of most of the tissue and masses of polynuclear leucocytes. Traces of the original tissue appeared in islands of villi, densely filled with blood corpuscles. Branched mycelium could be seen penetrating the necrotic and purulent foci. It varied somewhat in diameter and was more or less angular and dilated in places. Most of the filaments appeared empty, at least the staining did not reveal any contents. No fruiting bodies, either in the form of sporangia or chlamydospores, were found.

As stated above, the intercotyledonous areas of the affected region of the chorion were beset with hemispherical elevations about 2 to 4 mm. in diameter, which gave the membrane a coarse goose-skin appearance. The sections showed that over the elevations it was deprived of its epithelium and the subjacent, bared zone filled with dense masses of nuclear debris which projected somewhat and gave the surface its nodular outline. This lesion is similar to that found in the bared chorion when *Bacillus abortus* is the agent, as well as in cases associated with *Vibrio fetus*. It would seem as if any injury to or destruction of the epithelium leads to a movement of leucocytes towards the surface, under which they gather in dense groups and disintegrate.

The invasion of the fetal membranes by a mould presupposes some primary focus in the body of the dam, whence spores might enter the circulation and break through into the fetal cotyledons. Unfortunately nothing is known of the dam, since the cow was slaughtered in the routine work of the abattoir and only the pregnant uterus reserved. If the above view of the source of the infection is correct it would imply the existence of moulds in the lungs of the cow where the oxygen requirements are sufficient for the production of sporangia and spores.

The literature on pathogenic mucors is very meager as contrasted with that on *Aspergillus*. Lichtheim[2] isolated from moistened bread two species pathogenic for rabbits after intravenous injection of fairly large doses of spores, *Mucor rhizopodiformis* and *Mucor corymbifer*. The chief loci of germination and growth were the kidneys, the lymphoid tissue of the intestines, and the mesenteric nodes. Paltauf[3] soon afterwards found a mucor in focal lesions of the cerebrum, cerebellum, lungs, pharynx, and ileum of a man 52 years old. The mucor was not cultured. The dimensions given indicate that he had a much smaller type under observation than the types mentioned above. Lindt[4] isolated two additional species from moistened, incubated wheat bread which were pathogenic for rabbits after intravenous injection of spores. These were named *Mucor pusillus* and *Mucor ramosus*, both distinguishable from one another and from the species cultured by Lichtheim on account of the shape and dimensions of the spores. Hückel[5] isolated a mucor from a cerumen plug taken from the ear of a patient 28 years old. This he identified with *Mucor corymbifer*.

Biological and Pathogenic Characters.

The mould was readily cultivated at incubator temperature on ordinary nutrient agar, although an addition of 1 per cent dextrose increased the vigor of the growth. At 70°F. the growth was much

[2] Lichtheim, L., *Z. klin. Med.*, 1884, vii, 140.
[3] Paltauf, A., *Virchows Arch. path. Anat.*, 1885, cii, 543.
[4] Lindt, W., *Arch. exp. Path. u. Pharmakol.*, 1886, xxi, 269.
[5] Hückel, A., *Beitr. path. Anat. u. Physiol.*, 1886, i, 117.

slower. It presented the following characters. A dense, branching, silken, whitish mycelium spreads over the surface and, in tubes, penetrates vertically for a distance of 5 to 10 mm. into the depths of the agar. The mycelium varies from 4 to 10μ in diameter. It contains a slightly refractile, coarsely or finely granular cytoplasm. The branching of the mycelium was at no time observed to follow any definite scheme. It evidently depended on the condition of the substrate.

In addition to this adherent layer of interlacing filaments an abundant aerial woolly mass of filaments appeared within 48 hours. These were found empty and often collapsed later on, probably as a result of drying. Rhizoids, or radiations of short filaments from centers of growth on the agar surface, were found, from which fruiting hyphæ arise to bear at the free end the sporangia. These sporangiophores and the rhizoids can be distinguished from the vegetative mycelium by their brownish tint under a low power. The rhizoid gives origin to a short trunk which soon divides irregularly into a small number of sporangiophores.

The sporangia, barely visible to the unaided eye, become a grayish brown color as they ripen and appear almost black under a low power in transmitted light. They are subspherical, measuring transversely about 80μ and vertically about 64μ, although smaller heads occur. The columella seen after discharge of the spores projects as a spherical body from the funnel-shaped, expanding end of the supporting hypha. The slightly brownish spores are spherical and measure about 4.5μ, although some as small as 4μ and as large as 5μ may be seen.

The mycelium on the agar surface may form in places two transverse septa close together. The intervening walls of the filaments bulge and the segment thus isolated and containing a granular cytoplasm forms a rather thick wall showing later on a double contour. The filaments on both sides of this new body shrivel. In rare instances a series of such segregated and encysted masses of cytoplasm may form. These bodies, usually denominated chlamydospores (*Gemmen*, *Dauerzellen*), were quite numerous in all cultures. No zygospores were seen at any time. An examination of the literature indicates that Lichtheim's *Mucor rhizopodiformis*[2] comes nearest to

the species under investigation, although there are certain differences such as a colorless, slightly larger spore (5 to 6μ) in Lichtheim's culture.

No special studies were made upon the behavior of this species under different environmental conditions. This much was frequently observed, however, that the mould is very capricious in the production of sporangia. Apparently the same conditions at one time lead to a rapid, copious formation of sporangia with ripening of spores in 2 to 3 days, in another to none. The obvious interpretation is that slight unrecognized differences in the environment exercise a decisive influence. Evidently oxygen is an important factor, for the sealing of tubes, which reduces the oxygen tension through absorption of the oxygen by the culture medium and by the mould during the early stages of growth, interferes with spore production.

To determine whether spores of the mould would germinate and develop a mycelium in the organs of rabbits, the growth from a dextrose agar culture was thoroughly stirred about in sterile bouillon and filtered through five layers of sterile fine bandage material to remove fragments of mycelium and clumps of spores. The resulting feebly clouded, lightly brownish fluid was found with only isolated spores. Doses of 1.5 and 0.5 cc. were injected into an ear vein of two rabbits weighing 2,218 and 2,404 gm. respectively. The rabbit receiving the smaller dose was in appearance normal until the 8th day, when it was chloroformed. The weight had dropped from 2,404 to 2,286 gm. The spleen was a trifle large and congested. It was beset with numerous subcapsular, 0.5 mm., isolated or agglomerated, whitish, slightly projecting nodules. Both kidneys show the same kind of nodules, about 1 to 5 mm. apart. Some of these correspond to opaque whitish streaks passing through the medulla and continuing in slenderer radial lines to the pelvis. In the latter no changes are evident. Scattering nodules occur in the liver and one in a Peyer's patch. The appendix is free. Several mesenteric nodes contain large necrotic foci. In one kidney nodule, crushed between slides, branching mycelium could be traced for some distance. Bits of spleen, liver, and kidney tissue placed in agar slants developed a rich mycelium within 24 hours.

The second rabbit died unexpectedly on the 7th day. No disturbance of health had been noticed but the weight at death was only 1,740 gm. Lesions much smaller than in the first rabbit were found in spleen and kidneys. The spleen was small and pale. The cause of death was probably some intercurrent influenza, since the lungs were generally congested and edematous and the nostrils ringed with dry crusts. There was, however, no similar disease among the older stock animals and it may be that the resistance had been lowered by the mould.

Sections of tissue of the first rabbit fixed in Zenker's fluid presented a number of interesting details which can only be briefly enumerated. The lesions of the kidneys in the cortex were made up of dense collections of polynuclear leucocytes within areas in which the interstitial cells had also proliferated. The affected area included one or several glomeruli and the tubules immediately surrounding them. The origin of the lesion, whether in a glomerulus or the tubules, could no longer be determined, as the original structures were barely identifiable. The focus thus begun could be traced downward, the associated tubules being filled with masses of partly disintegrated cells, chiefly polynuclears. These plugs extend to the tip of the papilla. Here the pelvis contains a mass of polynuclears completely surrounding the papilla. Branched mycelium could be seen in all the involved tubules. It was most abundant in the cellular masses of the pelvis. The changes in the spleen were similar to those in the cortex of the kidney. In the mesenteric nodes the foci were so numerous as to coalesce and involve more than half the node. Mycelium was specially abundant. In the liver two kinds of lesions were present, collections of polynuclear leucocytes taking the place of destroyed liver tissue and much smaller foci composed of multinucleated cells, some closely resembling those of the tubercle. No formed elements could be made out within these giant cells, but it is probable that they contained mould spores. Microscopic foci were not found in the lungs although all the injected spores must have passed through these organs.

These two inoculations serve to place this mucor among Lichtheim's so called pathogenic species. There are certain minor differences between his results and the present, which might disappear in

larger series. The slightly lower pathogenic effect when compared with Lichtheim's tests may be due to the fact that the inoculations were made after the mould had been kept 23 months on culture media.

The source of the infection in the cow can only be conjectured. The two widely distributed mucors, not pathogenic for rabbits, *Mucor mucedo* and *Mucor racemosus*, occur frequently in dairy products,[6] and it is probable that the species under consideration may be found there and thus represent a possible source of the infection.

SUMMARY.

A mucor, closely resembling Lichtheim's *Mucor rhizopodiformis*, was isolated from the diseased chorion of a cow and from the lungs and digestive tract of the fetus. No other microorganisms were detected. The mucor was demonstrated in teased preparations from the fresh cotyledons as well as in sections of tissues suitably hardened. It produced focal lesions in rabbits following the intravenous injection of spores. The condition of the amniotic fluid and the contents of the rumen of the fetus justify the inference that premature expulsion was impending.

[6] Weigmann, H., in Sommerfeld, P., Handbuch der Milchkunde, Wiesbaden, 1909, 328.

EXPERIMENTS ON THE NASAL ROUTE OF INFECTION IN POLIOMYELITIS.

By SIMON FLEXNER, M.D., and HAROLD L. AMOSS, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, December 3, 1919.)

In this paper we shall describe experiments bearing on the intranasal route of infection in poliomyelitis. That the inciting microorganism or virus of poliomyelitis enters the central nervous system by way of the nasal passages is now generally believed. Moreover, it has been shown that this virus may be present on the nasal mucosa without inducing any signs of disease.

Both healthy and so called chronic carriers of the virus of poliomyelitis occur in man. Wide diversity of view prevails as to the frequency with which carriage of the virus arises and as to the period of persistence of the virus in the carriers. According to one group of observers (Wickman,[1] and Kling, Pettersson, and Wernstedt[2]), healthy and chronic carriers arise numerously during epidemics of poliomyelitis and actually exceed, possibly even many fold, the number of actual cases of the disease. Moreover, the virus may be very persistent in carriers who have recovered from an attack of the disease and be detectable by animal inoculation several months after all the acute symptoms have subsided (Kling, Pettersson, and Wernstedt). However, it should be remarked here that the virus is supposed to undergo gradual deterioration and thus fail in producing typical experimental poliomyelitis, although it is still capable of exciting atypical symptoms and lesions.

Another group of experimenters has come to quite opposite conclusions. Thus Flexner and Amoss[3] who employed excised tonsillar and adenoid tissue for inoculation did not find either the great frequency of occurrence or the long survival of the virus in convalescents implied in the preceding statements. On the contrary, while they found the tonsillar and other tissues infective for monkeys during the early period of the disease in man, they observed no effects, as a rule, from the inoculation of the tissues taken after the acute symptoms had subsided.

[1] Wickman, I., Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

[2] Kling, C., Pettersson, A., and Wernstedt, W., *Communications Inst. méd. État Stockholm*, 1912, iii, 5.

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1919, xxix, 379.

The point of difference involved is important, not only on the general basis of public health considerations, but also because of its bearing on our conceptions concerning the epidemiology of poliomyelitis. The morbidity of poliomyelitis even during severe epidemics is low. This circumstance has been accounted for by assuming either that a relative insusceptibility to the disease exists among general populations, which is no explanation at all, or that because of the wide dissemination of the virus during an epidemic and even in interepidemic periods, an unperceived active immunization of the community takes place. Insusceptibility then is due to specific protection or immunity.

In all instances in man and monkey in which an experimental inquiry has been made, it has been found that when active immunity exists, the blood carries neutralizing or destructive bodies for the poliomyelitic virus. No systematic study of the blood of exposed persons who have remained free from obvious poliomyelitis has been made. We conducted a number of tests of the blood of nurses, doctors, and others who had been repeatedly exposed during the severe epidemic in New York State in 1916, without, however, obtaining any clear and decisive results.

The kind of assumed protection just indicated would be general and specific. But experiments of Amoss and Taylor [4] have shown that another kind of potential protective mechanism is demonstrable in man. This device is local and depends upon the presence in the nasal membrane and its secretions of a substance, not yet defined, which possesses the power of neutralizing or otherwise destroying the virus of poliomyelitis. It is suggested that this local process may play an important part in determining the morbidity of epidemic poliomyelitis.

The experiments to be described in this paper relate to several aspects of the problem of intranasal infection in poliomyelitis and bear, therefore, on the preceding discussion. The first experiments to be given concern the question of the power of the nasal mucosa of the monkey to suppress the virus of poliomyelitis directly applied to it; or, in other words, the ascertaining of the period of survival of the virus on the nasal membrane.

[4] Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

Fate of the Virus Applied to the Nasal Mucosa.

An effective means of inducing infection in *Macacus* monkeys is to apply an active poliomyelitis virus to the nasal membrane on a cotton pledget. By virus in this connection is meant the comminuted spinal cord and medulla of a monkey which has suffered from acute experimental poliomyelitis.

Not all monkeys so treated acquire infection; indeed, the percentage of successful inoculations by the nasal route is smaller than by the brain or intracerebral route. The first question to present itself, therefore, is the fate of the virus in animals which may not succumb to intranasal inoculation.

It has already been shown that given an active virus, it can be detected in the nasal membrane by means of subinoculation. That is, if the nasal membrane carrying the virus is excised, ground with sterile sand, suspended in isotonic saline solution, and filtered through a Berkefeld candle, the filtrate will set up poliomyelitis in another monkey into which it is injected intracerebrally and intraperitoneally. The experiments to follow show that with a constant sample of the virus and a uniform mode of inoculation, the survival of the virus on the nasal membrane is irregular and individual.

The virus was one which has long been kept active by monkey passages, and the mode of application was by cotton pledget, which was allowed to remain in a naris for 2 or more hours. Upon removal, the animals were kept under close observation for varying lengths of time, their condition was noted, and the excised nasal membrane, after etherization of the selected animals, employed for obtaining the filtrate, as described above, for purposes of inoculating other monkeys.

Experiment 1.—May 25. *Macacus rhesus* A. Cotton pledget carrying the virus remained in a naris over night. 60 hours after the removal of the plug, the animal was killed with ether and the nasal mucosa excised. The filtrate prepared from this membrane was inoculated into *Macacus rhesus* B.

May 28. *Macacus rhesus* B. Received 2 cc. of the filtrate by intracerebral and 5 cc. by intraperitoneal injection. This animal remained well until June 3, when it showed excitement and an ataxic, uncertain gait. The symptoms extended rapidly, paralysis occurred, and the animal died on June 6. The lesions present in the spinal cord and medulla were typical of poliomyelitis.

This experiment shows, therefore, that it is possible to detect the virus by the methods employed at least 60 hours after its application to the nasal membrane. But other tests carried out simultaneously or subsequently on other monkeys killed at the expiration of 40 and 60 hours and 8 days after the removal of the pledget resulted negatively. Hence this experiment may be taken to indicate that the nasal mucous membrane of the *Macacus rhesus* possesses in some instances striking power of destroying or eliminating the virus of poliomyelitis energetically applied to it.

The property of the nasal mucosa to render ineffective, under certain circumstances, an otherwise efficient dose of the virus may be shown in still another way.

The manner of invasion of the central nervous system by the virus of poliomyelitis is still an open question. In view of the difficulties surrounding experimental infection by way of the blood, and the relative ease with which it is accomplished by way of the brain, nasal membrane, sciatic nerve, peritoneum, eye, and even subcutaneous tissue, Flexner suggested that in all instances the passage of the virus from the periphery to the center is ultimately by way of the nerves. According to this view, the virus applied to the nasal mucosa extends along the short olfactory nerve fibers to the brain and spinal cord. A certain amount of support for this mode of infection is supplied by the experiments of Landsteiner and Levaditi[5] and of Flexner and Clark,[6] in which after an intranasal inoculation the brain and cord of the monkeys were removed before any symptoms appeared and injected separately into other monkeys. Flexner and Clark noted that 48 hours after an intranasal inoculation the olfactory lobes but not the medulla and spinal cord might be infectious.

But illuminating as this experimental result is, it must be regarded as the exception rather than the rule. It happens also and perhaps much more frequently that after an intranasal inoculation the virus cannot be detected either in the mucous membrane or in any portion of the central nervous system. The following protocol illustrates this point.

[5] Landsteiner, K., and Levaditi, C., *Ann. Inst. Pasteur*, 1910, xxiv, 833.
[6] Flexner, S., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1912–13, x, 1.

Experiment 2.—Macacus rhesus. June 1. The cotton pledget carrying the active virus was permitted to remain in the naris for 24 hours. No symptoms had developed by June 5, when the animal was etherized, 88 hours after the tampon had been removed. The right middle turbinate, at the site of the tampon, showed a small hemorrhage into the mucous membrane.

The nasal mucosa and heavy suspensions of the olfactory lobes, postrolandic convolutions, medulla, and cervical and lumbar spinal cord were inoculated separately into the brains of other *rhesus* monkeys. In no instance was infection secured. The control *Macacus rhesus* in which the pledget remained for the same period became paralyzed on the 7th and died on the 9th day. The lesions of the central nervous organs were typical of poliomyelitis.

The virus used in this experiment was active and the procedure adequate. The difference in the results may be attributed to the power of the nasal mucosa in the one and not in the other animal to destroy the virus. This is the more probable explanation, although it is, of course, possible that at the expiration of the 88 hour period the increase of the virus was insufficient to flood the central nervous system so as to be detectable by the inoculation test.

The first view given is, however, supported by another experiment in which the cotton plug carrying the active virus was permitted to remain in the naris only 2 hours. One of the *Macacus rhesus* monkeys developed symptoms, became paralyzed, and the nervous organs showed typical lesions of poliomyelitis. The other showed no symptoms and was etherized on the 16th day. The nasal mucosa, olfactory lobes, and medulla were injected intracerebrally into three *rhesus* monkeys of which none developed symptoms.

Effects of Antiseptics.

The innate destructive property possessed by the nasal membrane for the virus of poliomyelitis may be regarded as a valuable defensive mechanism. The question has often been raised whether, during an epidemic of poliomyelitis, the application of antiseptics to the nasal mucosa is to be recommended. In the case of chronic meningococcus carriers, the suppression of that microorganism by the introduction of antiseptics directly into the nasopharynx has not been notably successful; and the meningococcus is apparently a much more fragile organism than the microbe of poliomyelitis.

There is a further important consideration. Now that it has been shown that the nasal membranes are themselves defensive, account needs to be taken of the action of antiseptic drugs upon the chemical substances in the membranes upon which their protective function depends. It is fortunate that in the case of poliomyelitis the effects of chemical antiseptics on the virus of poliomyelitis implanted on the nasal mucosa can be directly tested experimentally.

We already possess fair data of the effects of disinfectants on the virus *in vitro*. The effective chemicals chiefly studied are hydrogen peroxide (Flexner and Lewis), formaldehyde (Römer), thymol, potassium permanganate (Landsteiner and Levaditi), and still others. In the experiments to be given the only antiseptics employed were chloramine-T and the oily solution of dichloramine-T, as devised by Dakin and Dunham.[7, 8]

Two protocols only of this series of experiments will be given. In a few instances in which the virus was applied to the nasal mucous membranes, monkeys treated with the dichloramine-T did not become ill or paralyzed, but as in these cases the control animals also failed to come down, it was considered probable that the particular sample of the virus employed for inoculation was ineffective, or that all the animals used were refractory.

Experiment 3.—Macacus rhesus. Apr. 16, 5 p.m. Inserted tampon with virus into left naris. Apr. 17, 10 a.m. Removed tampon which was slightly blood-stained. Both nares washed with 1:1,000 chloramine-T solution in water, after which the dichloramine-T in oil was sprayed into the nostrils; twenty-five successive expulsions of the oily solution by hand pressure were made for each side. The spraying was repeated at 12 m. and 2, 4, and 6 p.m. Apr. 18. Spray every 2 hours from 8 a.m. until 6 p.m. Apr. 23. No symptoms had appeared until this date on which the animal showed excitement and paralysis of the left arm. Apr. 25. Animal generally paralyzed and prostrate. Apr. 27. Animal dying; etherized. The lesions in the central nervous organs were typical of poliomyelitis.

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[7] Dakin, H. D., and Dunham, E. K., A handbook on antiseptics, New York, 1917, 33.

[8] We are indebted to Dr. E. K. Dunham for the preparation used in our experiments and for advice as to the procedure to follow.

The conditions of the above experiment are severe. The tampon was left in the naris for 17 hours, and injury, as indicated by the blood staining, had been inflicted on the mucous membrane. In the next experiment the conditions are less severe, but the result was not essentially different.

Experiment 4.—Macacus rhesus. May 25. Oily dichloramine-T solution sprayed into nares at 2, 4, and 6 p.m. May 26, 8 a.m. Spray as before. 10 a.m. Inserted cotton plug carrying the virus into the left naris. 12 m. Removed tampon and applied the oily spray. Repeated the spray at 2, 4, and 6 p.m. May 29. Animal protects the left leg. June 3. Ataxic; excited. June 4. Extensive paralysis; prostrate. June 8. Dead. The spinal cord and medulla showed typical lesions of poliomyelitis.

The two experiments given do not, of course, show conclusively that the application of antiseptic fluids to the nasopharynx exercises no restraining influence on the multiplication and pathogenic action of the virus of poliomyelitis present there. The conditions of the experiments may well have been too severe to be readily comparable with those arising in man. But account must also be taken of the fact that monkeys sometimes resist the introduction of the virus by means of tampons without any aid to the defensive powers of the nasal membranes whatsoever. If so active an antiseptic agent as the chloramines may thus be ineffective, it would seem that even less could be expected of the indiscriminate chemical solutions often applied by sprays to the nasopharynx.

Blocking Infection via the Nasal Mucosa.

The mere plugging of the naris of a *Macacus rhesus* with a tampon carrying the active virus of poliomyelitis may not suffice to set up infection. The outcome is determined not only by the degree of activity of the sample of the virus, but also by the strength of the defensive mechanisms possessed by the particular animal. It has been shown that the nasal mucosa is protective, but it appears also that other and deeper mechanisms play a part in supporting or reinforcing the nasal defenses.

One of the deeper mechanisms is the meningeal-choroidal plexus complex, as pointed out by Flexner and Amoss.[9] The latter ascertained that an otherwise ineffective virus tampon could be rendered effective if the integrity of this complex was disturbed as, for example, by setting up within it a temporary chemical inflammation. Various mild chemical irritants suffice for this purpose, but sterile alien serum is highly satisfactory.[9] But the particular point which the next experiments illustrate is not so much the fact of the promotion of nasal infection by the method indicated as the means employed to block infection by way of the nares.

Flexner and Amoss[9,10] have shown also that the introduction of immune poliomyelitic serum by lumbar puncture into the subarachnoid space in monkeys suffices to prevent infection by way of the meninges, blood, naris, etc. The question which was now investigated was whether blocking of the nasal infection could be secured by means of the immune serum injected into the blood.

The first protocol given is that of an unsuccessful attempt to block infection by way of the nares by means of hexamethyleneamine. This drug does display some power of destroying or of inhibiting the development of the virus of poliomyelitis *in vivo* (Flexner and Clark[11]). But its inferiority to immune serum is great, and the experiment which follows can be viewed in that light and also as another control observation.

Experiment 5.—Macacus rhesus. Mar. 6. 2 cc. of sterile normal horse serum injected intraspinally. Mar. 7. 2 hour virus-carrying cotton tampon in naris. Mar. 9. 0.5 gm. of hexamethyleneamine in 10 cc. of water given by stomach tube twice a day. Treatment repeated daily for 6 days. Mar. 15. No symptoms. Mar. 16. Animal excited, somewhat ataxic, and protects the right arm. Mar. 22. Condition remained stationary until this date, when the paralysis involved both arms, back, and right leg. Mar. 28. Dead. The lesions present in the central nervous organs were typical of poliomyelitis.

Experiment 6. (*a*) *Control.—Macacus rhesus.* Feb. 8, 4 p.m. 2 cc. of normal horse serum injected intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. Feb. 18. Arms paralyzed; back and right leg weak. Feb. 19. Pros-

[9] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 525.
[10] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1914, xx, 249.
[11] Flexner, S., and Clark, P. F., *J. Am. Med. Assn.*, 1911, lvi, 585.

trate. Feb. 20. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(*b*) *Test.—Macacus rhesus*. Feb. 8, 4 p.m. 2 cc. of normal horse serum intraspinally. Feb. 9. 2 hour nasal plug carrying the virus. On removal the plug was slightly blood-stained. Feb. 12. 5 cc. of immune monkey serum, obtained by pooling the blood from several animals, injected intravenously. This animal was kept under close observation for 2 months during which time no symptoms arose.

Experiment 7. (*a*) *Control.—Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour virus-carrying plug in naris. May 16. 2 cc. of normal horse serum intraspinally. May 17. Repeated 2 hour nasal plug. May 18. 2 cc. of normal horse serum intraspinally. May 19. Repeated 2 hour nasal plug. May 23. Left facial and right arm paralysis. May 24. Prostrate. May 26. Etherized. The medulla and spinal cord showed typical lesions of poliomyelitis.

(*b*) *Test.—Macacus rhesus*. May 14. 2 cc. of normal horse serum injected intraspinally. May 15. 2 hour cotton plug carrying the virus in naris; 10 cc. of pooled monkey poliomyelitic immune serum intravenously. May 16. 2 cc. of normal horse serum intraspinally. May 17. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. May 18. 2 cc. of normal horse serum intraspinally. May 19. 2 hour plug with virus in naris; 10 cc. of pooled monkey immune serum intravenously. This animal developed no symptoms whatever during several months observation.

Experiment 8. (*a*) *Control.—Macacus rhesus*. June 4. 2 cc. of normal horse serum intraspinally. June 5. 2 hour cotton plug carrying virus in naris. June 6. 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-containing nasal plug. June 8. 2 cc. of normal horse serum intraspinally. June 9. 2 hour virus-carrying nasal plug. June 13. Animal weak; no definite paralysis. June 14. Dead. The medulla and spinal cord showed lesions of poliomyelitis.

(*b*) *Tests*.—Two *Macacus rhesus* monkeys. Procedure identical in both. June 4.2 cc. of normal horse serum intraspinally. June 5. 2 hour virus-carrying plug in naris. June 6. 10 cc. of pooled monkey poliomyelitic serum intravenously and 2 cc. of normal horse serum intraspinally. June 7. 2 hour virus-carrying nasal plug. June 8. 10 cc. of pooled immune serum intravenously and 2 cc. of normal horse serum intraspinally. June 9. 2 hour nasal plug with virus. June 10. 10 cc. of pooled immune serum intravenously. No symptoms of poliomyelitis developed during the period of observation which extended over several months.

The results of this series of experiments are clear and definite, and show conclusively that even under highly favorable conditions of susceptibility the infection of monkeys with the virus of poliomyelitis applied to the nasal mucosa can be prevented by passive immuniza-

tion of the body by way of the general blood. By this means, therefore, the effective passage of the virus from the nasal mucosa to the central nervous organs can be blocked.

The precise point at which the blocking takes place is in doubt. Two or three possibilities exist: First, after passage of the virus into the blood itself *en route* to the brain and spinal cord. This possibility is small, inasmuch as all the available evidence is against the virus of poliomyelitis reaching the nervous organs from the general blood (Flexner and Amoss[10]). Next, in the nasal mucosa itself, as the blood carrying the immune serum circulates through. There is no way of readily affirming or excluding this idea. It seems improbable, however, that the virus in the interstices of the tissue and especially in the olfactory nerves themselves would have been brought under sufficient influence of the immune serum to have been prevented from multiplying and inducing infection. Third, in the central nervous system itself. In our opinion, this last is the more probable site. All the conditions of the experiments are favorable to the passage of a certain amount of the immune serum into the subarachnoid space. Under the influence of the chemical irritant, both the choroid plexus and the meningeal vessels become more pervious to protein substances and hence to the immune bodies (Flexner and Amoss[12]). Once the immune bodies reach the subarachnoid space and mingle with the cerebrospinal fluid, infection with the virus of poliomyelitis injected into the blood or meninges themselves is prevented.

There is little doubt that the quantity of immune serum employed in some of the experiments was excessive. Experiment 6 shows that a single intravenous injection suffices to block the development of the virus. But in the experiments in which repeated nasal tampons were employed and in which several injections of normal horse serum were given, it was deemed advisable to maintain the concentration of the immune serum in the general blood. After all, the answer sought by the experiments was whether under conditions of severe inoculation and a highly favorable degree of susceptibility of the animal tested, blocking of the infection could be secured surely by way of the passively immunized general blood.

[12] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1917, xxv, 499; 1918, xxviii, 11.

SUMMARY.

1. The experiments given in this paper, notwithstanding their seeming diversity, relate to the conditions underlying the states of susceptibility and refractoriness to infection with the virus of poliomyelitis applied to the nasal mucosa.

2. Certain monkeys are highly refractory to inoculation *via* the nares with the virus of poliomyelitis, apparently in virtue of a power possessed by the nasal mucous membrane to destroy or otherwise render ineffective the virus applied to it.

3. This property of the nasal mucosa appears to be distinct from any specific protective substance active upon the virus which may occur in the blood.

4. An effective nasal mucous membrane prevents the passage of the energetically applied virus to the brain and spinal cord.

5. The virus of poliomyelitis energetically applied to the nasal mucosa will survive for an undetermined period of time upon an ineffective, but for a relatively brief period of time upon an effective membrane.

6. The protective power possessed by the nasal mucosa is not in itself adequate to prevent infection with the virus introduced upon it, since slight injury to such independent structures as the meningeal-choroid plexus complex favors the passage of the virus from the nose to the central nervous organs.

7. The normal nasal mucosa is, therefore, an invaluable defense against infection with the virus of poliomyelitis; and the number of healthy and chronic carriers of the virus is probably determined and kept down through the protective activities of this membrane.

8. Antiseptic chemicals applied to the nasal mucosa upon which the virus has been deposited exhibit no great protective action and are of doubtful value. Indeed, it is not impossible that to the extent to which they may affect unfavorably the destructive properties of the nasal mucosa, they may be even objectionable.

9. Infection with the virus of poliomyelitis applied to the nasal mucosa under conditions favorable to the extension to the central nervous organs and multiplication there may be blocked or prevented by the injection of poliomyelitic immune serum into the blood.

While the exact manner and site of attack of the immune serum upon the virus is somewhat conjectural, when all the available data are considered it seems probable that the meeting place of the virus and immune serum is in the subarachnoid space.

ETIOLOGY OF YELLOW FEVER.

X. Comparative Immunological Studies on Leptospira icteroides and Leptospira icterohæmorrhagiæ.

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In a previous paper[1] it was reported that serum from convalescent yellow fever patients has a more or less marked antagonistic effect upon *Leptospira icteroides* derived from certain cases of yellow fever in Guayaquil, as manifested by a positive Pfeiffer phenomenon in the peritoneal cavity of the guinea pig. In a few instances the serum protected the guinea pig from a fatal infection with the organism. A similar result was obtained with the serum of guinea pigs which had recovered from a non-fatal experimental infection with the leptospira.

In the present paper the question of immunity will be more fully discussed, particularly with regard to agglutination, lysis, complement fixation, Pfeiffer's reaction, etc., with immune sera prepared in rabbits and horses by repeated inoculations of *Leptospira icteroides*. Experiments have also been carried out to determine the relation between this organism and *Leptospira icterohæmorrhagiæ* of infectious jaundice by means of cross-immunity reactions *in vitro* and *in vivo*. In the *in vivo* experiments not only passive, but also active immunity has been taken into consideration. A part of this study has already been published in a paper dealing with the leptospira isolated from wild rats and mice in Guayaquil[2] and will not be repeated here.

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.
[2] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

Production of Immune Sera.

Monovalent immune sera for each of four strains of *Leptospira icteroides* were prepared in rabbits by injecting the animals intravenously with 2 to 4 cc. of rich live culture, on rabbit serum medium, several successive times at intervals of 7 to 14 days. The animals showed a definite febrile reaction on the 4th or 5th day after the first injection but no other symptoms. Subsequent inoculations produced no perceptible reaction in most of the animals, although some died suddenly, probably owing to the development of anaphylaxis as the number of inoculations increased.

Polyvalent immune serum was produced in a horse by injecting intravenously (jugular vein) gradually increasing amounts of rich live cultures (horse serum medium) of five strains (Nos. 1, 3, 4, 5, and 6) of *Leptospira icteroides*. 20 cc. of the mixture of cultures were given as the initial injection, and subsequent injections were increased up to 200 cc. This dose was maintained for most of the time during immunization. The first inoculation caused a rise in temperature to 40°C. on the 3rd and 4th days, with considerable swelling along the inoculated side of the neck. The animal lost its appetite during the period but regained its normal condition within 5 days. There was no jaundice at any time. Subsequent inoculations caused no perceptible reaction. During a period of 65 days the horse received 2,495 cc. of mixed live cultures in fifteen injections. The following protocol gives the schedule of immunization.

Horse 2.—Feb. 6, 1919, 20 cc.; Feb. 11, 40 cc.; Feb. 15, 60 cc.; Feb. 19, 125 cc.; Feb. 24, 150 cc.; Mar. 1, 200 cc.; Mar. 6, 200 cc.; Mar. 11, 200 cc.; Mar. 15, 200 cc.; Mar. 19, 200 cc.; Mar. 24, 200 cc.; Mar. 28, 200 cc.; Apr. 2, 200 cc.; Apr. 7, 200 cc.; Apr. 12, 300 cc. First bleeding on Apr. 19, 1919.

Effects of Immune Sera upon Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Monovalent immune sera were prepared in rabbits, as described, and experiments conducted to demonstrate the effects of such sera upon homologous and heterologous strains of *Leptospira icteroides* on the one hand and of *Leptospira icterohæmorrhagiæ* on the other.

The experiments were designed to throw more light on the relation that may exist, not only among different strains of these organisms, but also between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* as distinct varieties. The effects of an immune serum are manifold, but we confined our observations to the phenomena of agglutination, immobilization, and disintegration of the organism when mixed with the immune serum *in vitro* and that of the reaction of Pfeiffer following the simultaneous inoculation of the organism and an immune serum into the peritoneal cavity of guinea pigs. Complement fixation tests were also made.

Agglutination.—The technique employed for the agglutination tests was as follows:

Rich live cultures, grown at 26°C. for 2 to 3 weeks on rabbit serum medium (one part of serum plus three parts of 0.9 per cent sodium chloride solution), of each strain were selected. 1 cc. of each of a number of cultures was put into a series of small sterile test-tubes, and 0.2 cc. of the fresh immune serum to be tested was added to each tube. Controls with normal rabbit serum accompanied each series of experiments. The culture and immune serum were carefully mixed by gentle shaking and the tubes incubated at 37°C. (water bath) for 2 hours. Examinations of the contents were then made by means of the dark-field microscope.

With pronounced agglutination minute particles could be macroscopically detected. Another examination was made on the tubes after they had been left at room temperature for 96 hours, but the results were identical with those recorded after 3 hours, with the possible exception of a more granular appearance of some of the agglutinated cultures and in extreme instances a thin but definite grayish sediment at the bottom of the test-tubes.

In the present series of experiments there were available three monovalent antisera for *Leptospira icteroides*, comprising Strains 1, 5, and 6, and six for *Leptospira icterohæmorrhagiæ*, comprising the Japanese, British, American No. 1, Group 8, Group 11, and Group 30 strains. Against each of these nine monovalent immune sera were tested cultures of five different strains of *Leptospira icteroides*, Nos. 1, 3, 4, 5, and 6, and seven strains of *Leptospira icterohæmorrhagiæ*, Japanese, British, French, American No. 1, Group 8, Group 11, and Group 30.

A strong immune serum acting upon the homologous strain of *Leptospira icteroides* agglutinated the organisms quickly into rather large masses, in which they appeared tightly held together. Most of the organisms became immobile, gradually lost their elementary windings, and were soon transformed into stiff, irregularly granular filaments. If the serum was not strong enough to produce these changes, the agglutinated masses were for the most part degenerated, but with several apparently intact immobilized organisms at the periphery. In other instances the agglutinated masses contained a certain number of individuals which were still active, while in still others the organisms in agglutinated masses showed quivering motility in some part of their body. On the whole, agglutination is the first and more constant reaction observed and disintegration the secondary and less constant (Tables I and II).

Pfeiffer's Phenomenon.—To complete the observations, Pfeiffer tests were also performed with the immune sera and the various strains of *icteroides* and *icterohæmorrhagiæ*. The technique employed was that generally followed. 1 cc. of a given immune serum was mixed in a Petri dish with 1 cc. of a rich live culture and immediately injected into the peritoneal cavity of a normal guinea pig. The peritoneal fluid was withdrawn with a capillary pipette after 30 minutes and 2 hours and examined under the dark-field microscope (Tables I and II).

The reaction of Pfeiffer with the immune sera and homologous strains of *Leptospira icteroides* is prompt and complete. The organisms seem first to be agglutinated into large masses and then to be quickly disintegrated. The phenomenon may be complete within 15 minutes, so that no trace of the organisms can be seen in the peritoneal fluid. The same is true of *Leptospira icterohæmorrhagiæ* and the homologous immune sera. A decided increase of actively motile organisms was noticed in the guinea pig peritoneal cavity when a normal rabbit serum instead of a specific immune serum was used.

As shown in Table I, the five different strains of *Leptospira icteroides* reacted to each of the three monovalent immune sera. The intensity of agglutination and disintegration varied somewhat according to whether the strains were homologous or heterologous. Without

TABLE I.

Effects of Monovalent Anti-icteroides Sera upon Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Serum.	Strain of *Leptospira icteroides*.															Strain of *Leptospira icterohæmorrhagiæ*.																				
	No. 1.			No. 3.			No. 4.			No. 5.			No. 6.			Japanese.			British.			French.			American No. 1.			Group 8.			Group 11.			Group 30.		
	Agglutination.*	Disintegration.*	Pfeiffer reaction.†	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.
Immune serum for *Leptospira icteroides*, Strain 1.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
	+	+		+	+		+			+	+		+	+		+																			
	+	+		+			+			+			+																						
	+	+																																		
" " " " " " 5.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	+	+		+	+		+	+		+	+		+	+																					
	+	+		+	+		+			+	+		+																						
	+									+	+																									
" " " " " " 6.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	±	−	−	−	−	−	−	−	−
	+	+		+	+		+	+		+	+		+	+																						
	+			+	+		+	+		+			+	+																						
	+						+						+	+																						
Normal rabbit serum (control).	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* The varying degrees of agglutination and disintegration are recorded by plus signs vertically placed; their absence by a minus sign.
† + indicates positive; ± doubtful; − negative.

TABLE II.

Effects of Monovalent Anti-icterohæmorrhagiæ Sera upon Leptospira icterohæmorrhagiæ and Leptospira icteroides.

Serum.	Strain of *Leptospira icterohæmorrhagiæ*.																					Strain of *Leptospira icteroides*.																	
	Japanese.			British.			French.			American No. 1.			Group 8.			Group 11.			Group 30.			No. 1.			No. 3.			No. 4.			No 5.			No. 6.		
	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.	Agglutination.	Disintegration.	Pfeiffer reaction.
Immune serum for *Leptospira icterohæmorrhagiæ*, Japanese strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−
	+	+		+			+			+	+		+	+		+			+	+		+																
	+	+								+	+		+	+					+	+																		
	+	+								+	+		+	+					+	+																		
Immune serum for *Leptospira icterohæmorrhagiæ*, British strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	+			+	+		+	+		+			+			+			+																			
				+	+		+	+								+																						
				+	+											+																						
Immune serum for *Leptospira icterohæmorrhagiæ*, American Strain 1.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	±	−	−	−	+	−	±			
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	+	+								+	+		+	+																								
	+									+	+		+																										
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 8 strain.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	+	+		+	+		+			+			+	+		+	+		+	+																		
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exception the strongest reaction was obtained with the homologous and a less pronounced one with heterologous strains. Disintegration of the organisms was usually complete in the homologous but seldom so in heterologous combinations. Normal rabbit serum exerted neither an agglutinating nor a disintegrating influence upon any of the strains studied. On the contrary, the addition of normal rabbit serum to control tubes kept the organisms active for many days.

That these anti-*icteroides* sera did not agglutinate the various strains of *Leptospira icterohæmorrhagiæ* to any marked degree is also shown in this table. There were a few instances in which slight agglutination was observed, but none so marked as that which occurred with the strains of *icteroides*. In contrast to the results obtained with the *icteroides* strains, in no instance was there any disintegration of the *icterohæmorrhagiæ* organisms by an anti-*icteroides* serum. The Pfeiffer phenomenon was invariably positive with the anti-*icteroides* serum and the *icteroides* strains, but almost always negative when the anti-*icteroides* serum was tested with the *icterohæmorrhagiæ* strains. In two instances there was a suggestive reaction.

Table II presents the results obtained with six different monovalent anti-*icterohæmorrhagiæ* sera. The marked difference that exists between the *icterohæmorrhagiæ* strains and the *icteroides* strains is shown. Aside from slight variations, the *icterohæmorrhagiæ* strains reacted with the *icterohæmorrhagiæ* antisera quite generally and strongly and present a marked contrast to the *icteroides* strains, which reacted occasionally and never very strongly. The Pfeiffer phenomenon was positive in all combinations of the anti-*icterohæmorrhagiæ* serum with the *icterohæmorrhagiæ* group, but only occasionally and slightly with the *icteroides* group. The occurrence of a fairly marked agglutination in certain instances, such as, for example, anti-Japanese serum *versus* No. 1 *icteroides* strain or anti-group No. 11 serum *versus* No. 3 *icteroides* strain, is of considerable interest because of the occurrence of similar weak reactions among the combinations of anti-*icterohæmorrhagiæ* sera and certain heterologous *icterohæmorrhagiæ* strains. The only reliable differentiation in these instances would seem to be that of the Pfeiffer test. Beyond these few irregularities the behavior of the immune sera towards *icterohæmorrhagiæ* and *icteroides* seems to warrant the conclusion that the strains of

Leptospira icteroides and *Leptospira icterohæmorrhagiæ* form closely related but distinct groups.

Complement Fixation.—The technique used in the complement fixation tests was as follows:

Rich cultures of various strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* were grown on rabbit serum media, killed by placing the culture tubes in a water bath at 60°C. for 10 minutes, and then used as antigens in the complement fixation tests. Graduated quantities (0.1, 0.05, 0.02, 0.01, 0.005 cc.) of each of the monovalent sera were mixed with a uniform quantity of the antigen (a quantity which had been found to be non-anticomplementary in several doses when tested with 0.1 cc. of complement), and to each tube was added 0.1 cc. of fresh guinea pig serum as complement. The mixtures were brought up to a total volume of 1.5 cc. for each tube by the addition of 0.9 per cent saline solution. After incubation at 37°C. for 1 hour, 0.1 cc. of a 20 per cent suspension of washed sheep corpuscles and 0.1 cc. of anti-sheep amboceptor (rabbit serum), representing three hemolytic units, were added to each tube, the contents thoroughly mixed, and once more incubated at 37°C. for 30 minutes. The results were read after standing for 1 hour. The actual quantity of each antigen used was 0.1 cc. of the killed culture, which exhibited only a slight anticomplementary property when used alone in quantities of from 0.4 to 0.6 cc.

In the majority of instances the reaction was maximum with 0.1 cc. of immune serum, and all tests with 0.005 cc. gave a negative result. The readings of the reaction obtained with 0.1 cc. of the antigens and 0.1 cc. of the immune sera are recorded in Table III.

Complete fixation took place when the immune sera were mixed with the homologous strains, both in the case of *Leptospira icteroides* and in that of *Leptospira icterohæmorrhagiæ*. Occasionally, especially among the *icteroides* strains, the fixation was as strongly positive with one or the other of the heterologous strains as with the homologous. There were a number of instances also in which a more or less definite fixation occurred when the anti-*icteroides* sera were mixed with the *icterohæmorrhagiæ* strains or the anti-*icterohæmorrhagiæ* sera with the *icteroides* strains, some reactions being as strong as ±. Generally speaking, however, there was only a limited degree of cross-reaction between *icteroides* and *icterohæmorrhagiæ*.

Variations in the fixation reaction were rather marked among the different strains of *icterohæmorrhagiæ*, according to the combinations of the immune sera and heterologous strains, some of which showed only a feeble fixation (+) with certain sera.

TABLE III.

Complement Fixation Tests.

Serum.	Strain of *Leptospira icteroides*.					Strain of *Leptospira icterohæmorrhagiæ*.									
	No. 1.	No. 3.	No. 4.	No. 5.	No. 6.	Japanese.	British	French.	American No. 1.	American No. 2.	American No. 3.	Group 8.	Group 11.	Group 30.
Immune serum for *Leptospira icteroides*, Strain 1.	+*	+	+	+	+	+	−	−	−	−	−	−	+	−
	+	+	+	+	+									
	+			+										
	+			+										
Immune serum for *Leptospira icteroides*, Strain 5.	+	+	+	+	+	−	−	−	−	+	+	−	−	+
	+	+	+	+	+									+
	+	+		+										
	+			+										
Immune serum for *Leptospira icteroides*, Strain 6.	+	+	+	+	+	−	−	−	+	−	−	+	+	+
	+	+	+	+	+									
	+	+	+		+									
			+		+									
Immune serum for *Leptospira icterohæmorrhagiæ*, Japanese strain.	+	±	+	−	−	+	+	+	+	+	+	+	+	+
						+			+	+	+	+	+	+
						+								+
						+								
Immune serum for *Leptospira icterohæmorrhagiæ*, British strain.	−	−	−	−	−	+	+	+	+	+	+	+	+	+
						+	+	+					+	+
							+	+						
							+							
Immune serum for *Leptospira icterohæmorrhagiæ*, American Strain 1.	−	+	+	−	−	+	+	+	+	+	+	+	+	+
						+	+	+	+	+	+	+	+	+
									+		+			
									+		+			
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 8 strain.	−	−	−	−	−	+	+	+	+	+	+	+	+	+
						+	+	+	+	+	+	+	+	+
									+			+		+
												+		

TABLE III—Concluded.

Serum.	Strain of *Leptospira icteroides*.					Strain of *Leptospira icterohæmorrhagiæ*.									
	No. 1.	No. 3.	No. 4.	No. 5.	No. 6.	Japanese.	British.	French.	American No. 1.	American No. 2.	American No. 3.	Group 8.	Group 11.	Group 30.
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 11 strain.	−	+	−	−	−	+	+	+	+	+	+	+	+	+
		+				+		+	+	+	+	+	+	+
						+			+	+	+		+	
													+	
Immune serum for *Leptospira icterohæmorrhagiæ*, Group 30 strain.	−	−	−	−	+	+	+	+	+	+	+	+	+	+
						+		+	+	+	+	+	+	+
						+					+			+
						+								+

* Four plus signs vertically placed indicate no hemolysis, or complete fixation; three plus signs, about 25 per cent hemolysis; two plus signs, about 50 per cent hemolysis; one plus sign, about 75 per cent hemolysis. ± indicates almost complete hemolysis, and minus, complete hemolysis, or no fixation.

Protective Properties of Immune Sera against Leptospira icteroides.

Monovalent Immune Rabbit Sera.—Several monovalent immune rabbit sera, some specific for the *icteroides* strains and some for the *icterohæmorrhagiæ*, were tested for their protective properties against the experimental infection in guinea pigs with *icteroides* or with *icterohæmorrhagiæ*. For this purpose 1 cc. of culture of the strain to be tested was mixed with 1 cc. of immune serum and the mixture injected intraperitoneally into the guinea pig (Table IV).

It was found that Nos. 5 and 6 of the *icteroides* strains were prevented from causing any infection in guinea pigs when inoculated simultaneously, not only with their respective antisera, but also in combination with heterologous antisera. This would indicate that an immune serum prepared with one strain of *Leptospira icteroides* is equally protective against another strain of the same organism. That the protection was specific may be seen from the fact that these two strains of *Leptospira icteroides* caused either a fatal or a rather

TABLE IV.

Protective Properties of Monovalent Immune Rabbit Sera against Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Serum.	Strain of *Leptospira icteroides*.		Strain of *Leptospira icterohæmorrhagiæ*.			
	No. 5.	No. 6.	Japanese.	French.	Guayaquil No. 8.	Guayaquil No. 30.
Normal rabbit serum (control).	One guinea pig died in 7 and the other in 6 days.	Died in 6 days.	Died in 9 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Two guinea pigs died in 7 days. Typical lesions.	Two guinea pigs died in 8 days. Typical symptoms and lesions.
Immune Serum 945. Homologous with *Leptospira icteroides*, Strain 5.	Survived. No symptoms.	Survived. No symptoms.	Survived; severe infection with marked lesions.	Survived; severe infection.	Died in 9 days. Typical lesions.	Died in 11 days. Typical symptoms and lesions.
Immune Serum 942. Homologous with *Leptospira icteroides*, Strain 6.	Survived. No symptoms.	Survived. No symptoms.	Died in 9 days. Typical symptoms and lesions.	Survived; severe infection. Marked lesions.	Died in 8 days. Typical lesions.	Died in 10 days. Typical symptoms and lesions.
Immune Serum 911. Homologous with *Leptospira icterohæmorrhagiæ*, Japanese strain.	Died in 10 days. Typical symptoms and lesions.	Died in 10 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. Examination showed a few lung lesions.	Survived. No symptoms.	Survived. No symptoms.

Immune Serum 947. Homologous with *Leptospira icterohæmorrhagiæ*, British strain.	Died in 9 days. Typical symptoms and lesions.	Died in 12 days. Typical symptoms and lesions.	Survived. Slight lesions.	Survived. No lesions.	Survived. No symptoms.	Survived; mild infection.
Immune Serum 952. Homologous with *Leptospira icterohæmorrhagiæ*, American Strain 1.	Died in 11 days. Typical symptoms and lesions.	Survived; mild infection.	Survived; mild infection.	Survived. No lesions.	Survived; mild infection.	Survived.
Immune Serum 904. Homologous with *Leptospira icterohæmorrhagiæ*, Guayaquil Strain 30.	Survived; severe infection.	Died of intercurrent disease.	Survived. Slight lesions.	Survived. No lesions.	Survived. No symptoms.	"

severe infection in guinea pigs when mixed with normal rabbit serum or with one or another of the antisera produced with different strains of *Leptospira icterohæmorrhagiæ*. It is noteworthy that the period of incubation when anti-*icterohæmorrhagiæ* serum and *Leptospira icteroides* are combined is somewhat prolonged. For example, with Strain 5 of *icteroides* the control animals with normal rabbit serum died in 6 or 7 days, while the guinea pigs receiving the anti-*icterohæmorrhagiæ* sera died between 9 and 11 days after the inoculation. This is also true of Strain 6, with which death occurred in 6 days in the control animal and in 10 and 12 days in the animals injected with the anti-*icterohæmorrhagiæ* sera. The symptoms and lesions were typical in all instances in which an infection ensued.

The results obtained by reversing the combinations, that is by mixing different strains of *Leptospira icterohæmorrhagiæ* with anti-*icteroides* immune sera, show also an unmistakable specificity of the protection afforded by these immune sera. A clear-cut specific protection is shown in the experiments with the Japanese and Guayaquil strains of *icterohæmorrhagiæ*; these three strains were effectively neutralized by their homologous immune sera, but not by any anti-*icteroides* immune sera, although the sera seemed to delay death in some instances. The French strain of *icterohæmorrhagiæ* was least virulent and did not cause fatal infection in guinea pigs when any one of the immune sera was simultaneously inoculated. The examination of the surviving guinea pigs for the lesions, particularly those in the lungs, after 24 days showed that the guinea pigs which received the anti-*icteroides* sera had numerous old hemorrhagic foci in the lungs, while none or only a few foci were found in those which were inoculated with the anti-*icterohæmorrhagiæ* sera.

Polyvalent Immune Horse Sera.—A horse was immunized with a mixture of cultures of four strains of *Leptospira icteroides* for a period of 65 days, during which 2,495 cc. of the cultures were injected intravenously as described in the protocol above. At the same time another horse, which had once been immunized for a period of several months in 1918 with various strains of *Leptospira icterohæmorrhagiæ*, was injected again, with *icterohæmorrhagiæ* cultures, comprising nine strains: Japanese (one strain), American (three strains), French (one strain), British (one strain), and Guayaquil (three strains).

The serum from each horse was then tested for its protective property in guinea pigs against some of the representative strains of *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*.

The procedure consisted in injecting into the peritoneal cavity of guinea pigs a mixture of a given quantity of culture (or liver emulsion) of a strain with 1 cc. of immune serum, either full strength or diluted, as indicated in Tables V and VI.

For each dose of the serum two guinea pigs were used in order to determine its titer as closely as possible. Only one guinea pig, however, was used in determining the protective titers of the anti-*icteroides* serum against the *icterohæmorrhagiæ* strains or *vice versa*. The amounts of culture (or liver emulsion) used in testing the corresponding antiserum were such as to approximate 500 minimum lethal doses, while for a cross-titration usually about 50 minimum lethal doses, or even 10 minimum lethal doses were chosen. The reason for reducing the number of minimum lethal doses in cross-protection experiments was that in several preliminary tests a larger quantity of culture was found not to be influenced to any great extent by a heterologous antiserum.

The anti-*icteroides* serum protected guinea pigs against approximately 500 minimum lethal doses of *Leptospira icteroides* in doses of 0.001 (for Strain 5) and 0.0001 cc. (for Strain 6). In other words, 1 cc. of this serum neutralizes about 500,000 to 5,000,000 minimum lethal doses of *Leptospira icteroides*, according to the degree of virulence of the culture. On the other hand, at least 1 cc. of the same serum was required to protect guinea pigs against 10 to 50 minimum lethal doses of the Japanese and French strains of *Leptospira icterohæmorrhagiæ*. 0.1 cc. of the serum averted a fatal outcome but failed to prevent wholly the infection. The difference in the protective efficacy of the anti-*icteroides* serum against *icteroides* and *icterohæmorrhagiæ* is striking.

The anti-*icterohæmorrhagiæ* serum exhibited also a marked specific protective property for the *Leptospira icterohæmorrhagiæ* strains, neutralizing at least 5,000,000 (for the French) to 500,000 (for the Japanese) minimum lethal doses per 1 cc. Its effect upon the *icteroides* strains was in each case feeble but distinct, since it protected guinea pigs completely against 50 minimum lethal doses per 1 cc.

TABLE V.

Protective Properties of Polyvalent Anti-icteroides Serum against Leptospira icteroides and Leptospira icterohæmorrhagiæ.

Anti-*icteroides* serum.	Strain of *Leptospira icteroides*.		Strain of *Leptospira icterohæmorrhagiæ*.	
	No. 5. 0.1 cc. of culture (about 500 M. L. D.).	No. 6. 0.1 cc. of culture (about 500 M. L. D.).	Japanese. 0.1 cc. of liver emulsion (about 10 M. L. D.).	French. 0.1 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 7 days. Typical symptoms and lesions.	Died in 7 days.	Died in 7 days.	Died in 9 days.
0.000001	Died in 9 days. Typical symptoms and lesions.	" " 10 "		
0.00001	Survived.	" " 9 "	Died in 6 days.	Died in 10 days.
0.00001	Died in 9 days.	Survived.		
0.0001	" " 9 "	"	Survived (!).	Died in 8 days.
0.0001	Survived.	"		
0.001	"	"	Died in 14 days.	Died in 13 days.
0.001	"	"		
0.01	"	"	Died in 10 days.	Died in 13 days.
0.01	"	"		
0.1	"	"	Survived. Had fever.	Survived. Had fever.
0.1	"	"		
1.0	"	"	Survived. Had fever.	Survived. Had fever.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 7 days. Typical symptoms and lesions.	Died in 8 days.

and prevented death, but not infection, when used in a dose of 0.1 cc. It will be seen, therefore, that the respective polyvalent antisera exert a powerful annihilating effect in guinea pigs upon their corresponding type organisms, but there also exists an undeniable, though

feeble, cross-protective reaction, which may be explained by assuming that the two groups of organisms are not altogether alien but are closely related to each other; they may even constitute two subspecies or races.

TABLE VI.

Protective Properties of Polyvalent Anti-icterohæmorrhagiæ Serum against Leptospira icterohæmorrhagiæ and Leptospira icteroides.

Anti-*icterohæmorrhagiæ* serum.	Strain of *Leptospira icterohæmorrhagiæ*.		Strain of *Leptospira icteroides*.	
	Japanese. 0.1 cc. of culture (about 500 M. L. D.).	French. 1 cc. of culture (about 500 M. L. D.).	No. 1. 0.1 cc. of culture (about 50 M. L. D.).	No. 5. 0.01 cc. of culture (about 50 M. L. D.).
cc.				
0.000001	Died in 6 days.	Died in 10 days.	Died in 8 days.	Survived (!).
0.000001	" " 7 "	" " 13 "		
0.00001	" " 6 "	Survived.	Died in 7 days.	Died in 8 days.
0.00001	" " 11 "	Died in 11 days.		
0.0001	Survived.	Survived.	Died in 8 days.	Died in 8 days.
0.0001	Died in 8 days.	"		
0.001	Survived.	"	Died in 7 days.	Died in 9 days.
0.001	"	"		
0.01	"	"	Died in 8 days.	Died in 15 days.
0.01	"	"		
0.1	"	"	Survived; fever.	Survived; mild jaundice.
0.1	"	"		
1.0	"	"	Survived.	Survived.
1.0	"	"		
Normal horse serum 1.0 cc. (control).	Died in 7 days.	Died in 7 days.	Died in 6 days.	Died in 7 days.

Active Immunity.

Guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*. It sometimes happens that a culture which kills guinea pigs of average susceptibility in a dose of about 0.0001 cc. occasionally

fails to produce a fatal infection in a guinea pig in a dose as large as 0.01 or 0.1 or even 1 cc. Such refractory guinea pigs are rarely met with, but the fact that there exist certain unusually resistant individuals is of great interest. It has also been found that there are exceptionally susceptible individuals which respond to infection with an attenuated culture which no longer attacks the average guinea pig. For example, with certain cultures of *Leptospira icteroides* which had been repeatedly subcultured without passage through the guinea pig for many months, only one out of several animals inoculated with the same culture may come down with typical symptoms. In fact, when the first attempt to restore the virulence of the culture by animal passage failed, a second or third attempt with four or five guinea pigs each time was necessary to obtain a single positive result. These facts furnish possible explanations for certain paradoxical results which are sometimes encountered in determining the state of immunity.

In a discussion of active immunity we may distinguish between that which arises from recovery from a genuine infection and that which follows the inoculation of killed organisms. Animals which, after receiving an inoculation of a sublethal dose of a live culture, do not react definitely, may acquire a state of immunity similar to that of vaccinated animals.

The results with the guinea pigs which had recovered from a more or less pronounced infection after the inoculation of a culture or blood derived from a guinea pig dying of the typical infection with *Leptospira icteroides* will first be described. The tests consisted in the Pfeiffer phenomenon and the effect of the inoculation of different cultures in case of a negative Pfeiffer reaction.

Series 1.

Four guinea pigs were actively immunized with Strain 5 of *Leptospira icteroides*.

Guinea Pig Ch 1.—Nov. 27, 1918. Received 1 cc. of citrate blood from a guinea pig which had been infected with Strain 5 of *Leptospira icteroides* and showed the typical symptoms of *icteroides* infection. After the usual course of infection (fever, slight icterus, albuminuria) the animal became well within 2 weeks. Dec. 19. Received 1 cc. of culture of the same strain. No symptoms followed. Jan. 6, 1919. Received another injection of culture of the same strain, without any perceptible effect.

Pfeiffer Reaction.—Jan. 18. A rich culture of the same strain (1 cc.) was injected intraperitoneally. A prompt positive reaction was obtained. Jan. 22. The Pfeiffer reaction was tested with Strain 6, 1 cc. of a rich culture being used. Result positive. On the same day, 4 hours later, another Pfeiffer test was made on this animal with the Japanese strain of *Leptospira icterohæmorrhagiæ*, 1 cc. of culture being used. Result negative. This animal died in 23 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 2.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Tested with 1 cc. of culture of the same strain. Result positive. Jan. 22. Pfeiffer test with 1 cc. of Strain 6 culture was positive. The same day, 4 hours later, tested with 1 cc. of French strain of *Leptospira icterohæmorrhagiæ*. Result negative. This animal died in 14 days with the typical symptoms of *icterohæmorrhagiæ* infection.

Guinea Pig Ch 3.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. Test with 1 cc. of the same strain was positive. Jan. 22. Test with 1 cc. of the British strain of *Leptospira icterohæmorrhagiæ* was doubtful. The animal survived.

Guinea Pig Ch 4.—The procedure with this animal was similar to that with Guinea Pig Ch 1.

Pfeiffer Reaction.—Jan. 18, 1919. With 1 cc. of culture of the same strain test was positive. Jan. 22. With 1 cc. of culture of Strain 6 of *Leptospira icteroides* test was positive. The animal survived.

Series 2.

Three guinea pigs, out of a large number inoculated with Strain 6 of *Leptospira icteroides*, which suffered more or less severe infection and eventually recovered, were reinoculated with a culture of the same strain once or twice (as described in the protocols below) afterwards, but showed no characteristic symptoms, except a rise of temperature for a day in one (Guinea Pig C 2). Hence they were completely immune to the same strain and were used to test their resistance to certain other strains, including the Japanese strain of *icterohæmorrhagiæ*.

Guinea Pig C 1.—Dec. 3, 1918. Received 1 cc. of Strain 6 culture of *Leptospira icteroides*. The animal had typical fever and a trace of jaundice, but recovered in about 9 days. Two more injections of 1 cc. of the same culture were given, one on Dec. 19, and another on Jan. 6, 1919.

Pfeiffer Reaction.—Jan. 18. With 1 cc. of a rich culture of the same strain, a prompt and positive reaction. Jan. 22. Inoculated with 1 cc. of a rich culture of Strain 5 of *Leptospira icteroides*. Pfeiffer reaction positive. No symptoms within 1 month.

Guinea Pig C 2.—Dec. 11, 1918. Received 1 cc. of blood from a guinea pig showing typical symptoms (fever and slight jaundice). The animal eventually

recovered. Jan. 6, 1919. 1 cc. of a rich culture of the same strain was given. There was slight fever for a day, but no infection followed.

Pfeiffer Reaction.—Jan. 18. 1 cc. of a culture of the same strain was inoculated intraperitoneally, but no organism could be found in the peritoneal exudate after 30 minutes. Jan. 22. The animal was tested again for the Pfeiffer reaction, with 1 cc. of a rich culture of Strain 1 of *Leptospira icteroides*. There was a complete positive reaction within 30 minutes, and no infection followed the inoculation.

Guinea Pig C 3.—This animal was immunized in the same way as Guinea Pig C 2.

Pfeiffer Reaction.—Jan. 18, 1919. 1 cc. of a rich culture of the same strain was injected intraperitoneally. Reaction prompt and complete. Jan. 22. 1 cc. of a rich culture of the Guayaquil strain, Group 30, of *Leptospira icterohæmorrhagiæ* was inoculated intraperitoneally. When examined after 2 hours the organisms were partially agglutinated, but most of them were actively motile. On the 4th day the temperature rose and remained above normal for 3 days. There was a slight jaundice on the 9th and 10th days, which soon faded. The animal survived. In this instance there existed a mild infection with the *icterohæmorrhagiæ* strain.

Series 3.

Nov. 27, 1918. Three guinea pigs were inoculated with a culture of the Japanese strain of *Leptospira icterohæmorrhagiæ*. A second injection was given on Dec. 19, and a third on Jan. 6, 1919. The animals showed a definite but mild infection after the first inoculation, but recovered. No symptoms followed the second or third injection of the same strain.

Guinea Pig J 1. Pfeiffer Reaction.—Jan. 18, 1919. With a culture of the Japanese strain, test positive. The animal remained well.

Guinea Pig J 2. Pfeiffer Reaction.—Jan. 18, 1919. With a rich culture of Strain 5 of *Leptospira icteroides*, test negative. The animal survived, passing through a moderately severe *icteroides* infection.

Guinea Pig J 3. Pfeiffer Reaction.—Jan. 18, 1919. Pfeiffer reaction with a rich culture of Strain 6 of *Leptospira icteroides* not clear-cut. There was a tendency to formation of agglomerated masses, without immobilization or lysis of the organisms. The animal showed mild but typical symptoms of *icteroides* infection after 13 days, but eventually recovered.

Supplementary Experiment with Anti-icterohæmorrhagiæ Serum.

Jan. 16, 1919. A number of guinea pigs were inoculated with mixtures of a polyvalent anti-*icterohæmorrhagiæ* horse serum prepared by Inada and Ido and different strains of *Leptospira icterohæmorrhagiæ* and *Leptospira icteroides*, with a view to determining the protective property of this serum against the *icterohæmorrhagiæ* as well as the *icteroides* strains. 1 and 0.1 cc. of the serum were used, mixed with 0.5 cc. of a culture of each strain, and inoculated at once into the peritoneal cavity

of guinea pigs. The protective titer of the serum had previously been tested against the Japanese strain of *Leptospira icterohæmorrhagiæ* and the serum had been found to neutralize 1 cc. of the culture in a dose of 0.001 cc. The result of the present experiment showed that the guinea pigs which received the immune serum and cultures of the Japanese, French, American, and Guayaquil strains of *icterohæmorrhagiæ* survived, while the control animals without the serum died in 6, 9, 8, and 7 days respectively. There were no symptoms observed in the surviving animals at any time; they were completely protected by the serum in the doses given (1 and 0.1 cc.). All the guinea pigs inoculated with a culture of the British strain of *icterohæmorrhagiæ* with or without (one control) the addition of the immune serum survived; that is, the culture employed was apparently avirulent.

The result obtained with the strains of *Leptospira icteroides* was somewhat surprising. The strains used in this group were Nos. 5 and 6, which killed the control animals in 5 and 10 days respectively Both cultures, however, produced only a temporary febrile reaction when injected together with 1 cc. of the serum and a moderately severe but non-fatal infection with 0.1 cc.

All the guinea pigs surviving in this series of experiments were subjected on Feb. 10, 25 days after the first inoculation, to immunity tests with cultures of various strains. The guinea pig which had received on Jan. 16 the mixture of the Japanese strain and 1 cc. of anti-*icterohæmorrhagiæ* serum escaped infection and was inoculated with 0.5 cc. of a culture of Strain 5 of *Leptospira icteroides* on Feb. 10. It died with typical symptoms of *icteroides* infection in 7 days. The guinea pig which escaped infection with the mixture of the French strain and 1 cc. of anti-*icterohæmorrhagiæ* serum on Jan. 16 and was injected with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 died in 5 days with typical symptoms of *icteroides* infection. The guinea pig which escaped infection with the mixture of American Strain 1 and 1 cc. of anti-*icterohæmorrhagiæ* serum on the first injection and was inoculated with 0.5 cc. of Strain 5 of *icteroides* on Feb. 10 finally recovered after a moderately severe *icteroides* infection.

As already mentioned, the guinea pigs which were inoculated on Jan. 16 with 1 and 0.1 cc. of anti-*icterohæmorrhagiæ* serum, together with the cultures of Strains 5 and 6 of *Leptospira icteroides*, had a temporary febrile reaction or a moderately severe infection. These animals were injected on Feb. 10, 25 days later, with a culture of the Japanese strain. All except one, which showed only slight lesions in the lungs, died with the typical symptoms of *icterohæmorrhagiæ* infection in 8 to 9 days. This would seem to indicate that these animals were protected by the anti-*icterohæmorrhagiæ* serum from the fatal outcome of the *icteroides* inoculations, but they were not rendered immune against the Japanese strain of *icterohæmorrhagiæ* when tested 25 days afterwards. Also the anti-*icterohæmorrhagiæ* serum injected on the first occasion had no perceptible protective action against this strain after a period of 25 days.

SUMMARY AND CONCLUSIONS.

It has been previously reported[3] that a filterable microorganism belonging to the genus *Leptospira* has been recovered from the blood or organs of human beings suffering from the disease known as yellow fever in Guayaquil, and that the organism, which has been termed *Leptospira icteroides*, induces in certain experimental animals the characteristic symptoms and lesions observed in the patients from whom it was isolated. It has also been previously shown[1] that the serum from patients recovering from an attack of yellow fever in Guayaquil had the power to agglutinate and dissolve the organism when introduced into the peritoneal cavity of a normal guinea pig (Pfeiffer phenomenon). Moreover, the guinea pigs which had once been inoculated with the blood of yellow fever patients without succumbing to the infection, notwithstanding the fact that they had shown a definite febrile reaction after 4 to 5 days, were found to be refractory to a subsequent inoculation of a culture of *Leptospira icteroides*.[4] All these observations pointed to the possible relation of this organism to the disease known as yellow fever in Guayaquil. The demonstration of the filterability of the organism[5] and the transmission of the infection with the same organism by *Stegomyia calopus*[6] have further strengthened the probable etiological significance of the organism in yellow fever.

It was by no means a simple problem to determine the relation existing between *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*. An experiment reported in a previous paper seemed to justify the view that the two leptospiras[7] are closely related but not identical, yet it was necessary to exhaust various other modes of differentiation before the distinction between them was firmly established. The present paper continues this phase of the inquiry in further detail.

There have been taken up here the phenomena of agglutination, the reaction of Pfeiffer, complement fixation, the protective properties of various monovalent and polyvalent immune sera, and active

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585; xxx, 87.
[4] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 1.
[5] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.
[6] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.
[7] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 95.

immunity. As the result of experiments in connection with these immunity phenomena the following data are presented.

Monovalent immune sera prepared by several successive injections in an animal naturally refractory to *Leptospira icteroides* possess the power to agglutinate *in vitro* not only the homologous strains, but also all other strains of *icteroides* tested. On the other hand, a slight effect, or none at all, has been observed when these immune sera have been mixed *in vitro* with various strains of *Leptospira icterohæmorrhagiæ*. A similar relation exists between the monovalent anti-*icterohæmorrhagiæ* sera and the various strains of *Leptospira icteroides*; that is, there is a slight agglutinating effect in some instances upon the *icteroides* strains, but it is never so strong as that occurring in tests against the *icterohæmorrhagiæ* strains. The Pfeiffer reaction gave a sharper differentiation between the two groups, for in most instances the phenomenon was specific for the group. There were occasional doubtful reactions, but not enough to warrant a confusion of the two groups.

Polyvalent immune sera, one specific for *icteroides*, and the other for *icterohæmorrhagiæ*, showed a high titer of neutralizing power for the cultures of the homologous groups. It was found, however, that the action of the sera is by no means absolutely specific, because the injection of a sufficient amount of the anti-*icteroides* serum apparently prevented a fatal outcome in a guinea pig inoculated with multiple minimum lethal doses of a culture of *Leptospira icterohæmorrhagiæ*, and *vice versa*. The specificity of the serum was demonstrated only when it was used in smaller quantities.

More or less specificity was shown by the complement fixation reaction, but it was not absolute. Weak fixation occurred when the anti-*icteroides* serum was mixed with one or the other of the *icterohæmorrhagiæ* strains and *vice versa*, and strong fixation occurred only when the antiserum was mixed with one of the *icteroides* strains. The question naturally arises whether or not this apparent specificity is due to the homology of the serum and not altogether to a difference in genus of the strains. In other words, it is justifiable to question whether all these variations in the degree of intensity of the reaction are not due to strain variations of the same genus. This question is not finally settled by the present investigation, in which only four

icteroides and nine *icterohæmorrhagiæ* strains have been carefully studied. Nevertheless, on the basis of the findings with these thirteen strains, it seems probable that *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ* are closely allied but are nevertheless distinct in their immunological reactions. Perhaps the difference between the two may amount to that between subspecies or races. It has been pointed out earlier that the pathogenicity of the two is also distinct, inasmuch as *icteroides* produces chiefly icterus and nephritis and *icterohæmorrhagiæ* hemorrhage and nephritis, the icterus being less and the hemorrhage more prominent in the evolution of the latter infection.

In the study of active immunity—exclusive of vaccination—difficulty has been experienced in the evaluation of the results, owing to the existence of natural resistance to infection among guinea pigs. A guinea pig may recover from the inoculation of *Leptospira icteroides* and then resist a subsequent inoculation with a virulent strain of *Leptospira icterohæmorrhagiæ*, a condition simulating that brought about by the identity of the two organisms. However, the refractoriness of such an animal to *icterohæmorrhagiæ* may be due to its natural immunity to it. In the present study, therefore, only those guinea pigs were selected which had reacted typically—though in mild degree—to the *icteroides* infection, in order to determine whether they were subsequently immune to the inoculation of *icterohæmorrhagiæ*. Indeed, by this mode of experimentation it was found that the guinea pigs which had once passed through an attack of the *icteroides* infection were absolutely immune to a second infection with the same organism but reacted severely and sometimes fatally to a later inoculation of *icterohæmorrhagiæ*. Although there were a number of instances in which a previous infection with *icteroides* did not confer any perceptible immunity upon the guinea pigs against *icterohæmorrhagiæ*, another group of guinea pigs showed a considerable resistance to the *icterohæmorrhagiæ* infection as compared with those which had never been inoculated with *icteroides*. There is not much doubt, therefore, that an *icteroides* attack brings about, in some instances at least, a certain degree of resistance to the *icterohæmorrhagiæ* infection. Hence the study of the phenomena of active immunity strongly indicates that *icteroides* is closely related immunologically to *icterohæmorrhagiæ*.

ETIOLOGY OF YELLOW FEVER.

XI. Serum Treatment of Animals Infected with Leptospira icteroides.

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The high potency attained by a polyvalent immune serum for *Leptospira icteroides* derived from the horse, as revealed in previous experiments on guinea pigs,[1] indicated the possibility that such a serum might be advantageously employed in the treatment of patients suffering from an infection with that organism. In order to ascertain whether or not the serum can exert a beneficial influence upon the course of the infection, several series of experiments were planned in which the guinea pigs were first inoculated with multiple minimal lethal doses of *Leptospira icteroides* culture and then treated with the immune serum at varying intervals afterwards.

Emphasis has been laid upon the fact that guinea pigs vary considerably in their susceptibility to *Leptospira icteroides*,[2] and instances have been cited[1] in which some animals survived after the injection of a large amount of culture, while some succumbed to smaller amounts. Irregularities of this nature were to be anticipated in the present series of experiments, but this source of error was eliminated as much as possible by using the serum in several graduated doses on a corresponding number of animals for a number of days in succession. In some of the earlier series tests were rendered unsatisfactory by the use of a culture which, from unknown causes, failed to kill the control animals, notwithstanding the fact that the same culture had been highly virulent when tested a fortnight previously. In another series the virulence of the culture employed was such that the control

[1] Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 135.
[2] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 585.

guinea pigs died within 5 or 6 days after the injection of the culture, and the time for treatment was comparatively brief.

The serum was injected intraperitoneally in amounts of 0.001, 0.01, 0.1, and 1 cc. at intervals of 1, 24, 48, 72, and 96 hours, and even 5, 6, and 7 days, if the animals still lived, after the inoculation of the culture. In the earlier experiments two injections a day of each dose were given (10 a.m. and 4 p.m.), but this practice was soon abandoned in favor of a single daily injection.

In determining the effect of the immune serum upon the infection, the temperature and other characteristic clinical symptoms were noted, and the extent of the lesions was ascertained by killing the surviving animals at a later period, when they were regarded as convalescent. The lesions chiefly considered, although not a wholly reliable index of the severity of the infection, were the hemorrhagic foci in the lungs.

In a series of therapeutic experiments in which the control guinea pigs died within 5 to 6 days, a culture of Strain 5 of *Leptospira icteroides* being employed, it was found that when injections of the immune serum were begun within 24 hours from the time of the experimental infection (intraperitoneal), no symptoms or lesions developed, provided the animal had received more than 0.001 cc. of the serum. If the treatment was begun 48 hours after infection, it was necessary to inject more than 0.01 cc. in order to prevent their development. At the end of 72 hours, when some of the guinea pigs had begun to show a rise of temperature, more than 0.1 cc. of the serum was necessary to check the progress of the infection, although some animals treated with 0.01 cc. also recovered. After 96 hours, when most of the animals had a high temperature, and some had begun to show a trace of jaundice, the injection of more than 0.01 cc. had a marked influence. The temperature came down to 100–102°F. by the following morning, and no jaundice afterwards appeared in these animals, which recovered within a week. A few guinea pigs, which were slightly icteric at the time of injection of the serum, became more deeply jaundiced on the following day and remained so for a few days, when they began to convalesce. No guinea pig treated within 96 hours with more than 0.01 cc. of serum died.

At the end of 5 days most of the animals which had not been treated with the serum showed jaundice and a decline in temperature, signs of approaching death within 24 hours. A few animals died in the afternoon of that day. To several of these critically ill guinea pigs 0.1 to 1 cc. of the serum was given twice, at 10 a.m. and at 4 p.m., but none of them seemed to be influenced by the injection. Some died on the same day, others on the following day, showing all the typical symptoms. At the end of 6 days several animals which still remained alive without the serum were treated with two doses of 1 cc., but none was saved from death on the same day.

It was found from this series of experiments that the progress of jaundice, after it had existed for 24 hours, could not be checked by the injection of the immune serum.

All the surviving animals were later killed for examination of the lung lesions. The animals treated with a sufficient amount (0.001 cc. or more) within 24 hours showed no lesions, or at most only a few hemorrhagic spots in the lungs. On the other hand, fairly numerous old hemorrhagic foci were found in the lungs of those which had been treated at the end of 48 hours, indicating an infection aborted through the action of the serum. The lung lesions were decidedly more diffuse and numerous in the guinea pigs which received the serum at the end of 72 and 96 hours, although none succumbed to the infection. In the animals which died following treatment with the serum later than 5 days after infection the symptoms and lesions were typical of the infection and indistinguishable from those in the control animals which died without receiving any serum.

In Table I is given another series of experiments carried out with Strain 6 of *Leptospira icteroides*. This culture killed control animals in doses of 0.001, 0.01, and 0.1 cc. within 13, 10, and 7 days respectively, although one guinea pig inoculated with 1 cc. of the culture escaped death after a severe infection. The amount of culture employed throughout the series was 0.5 cc. and was given intraperitoneally. The injections of serum were begun 1 hour after the inoculation and repeated daily for 7 days, in doses of 0.01, 0.1, and 1 cc.

As the table shows, no infection, so far as external manifestations are concerned, took place in any of the guinea pigs injected with the

TABLE I.

Effect of Polyvalent Immune Serum on Experimental Infection with Leptospira icteroides.

June 13, 1919. Thirty-seven guinea pigs were inoculated intraperitoneally, each with 0.5 cc. of Strain 6 of *Leptospira icteroides* culture, representing at least 500 minimal lethal doses. Graduated quantities (0.01, 0.1, and 1 cc.) of Immune Horse Serum 2 were given to the inoculated guinea pigs at intervals of 1, 24, 48, 72, and 96 hours, etc. The object of the experiment was to ascertain the minimal amount of the immune serum which prevents a fatal infection and the maximal time within which the inoculated animals may still be saved by the injection of the serum.

Guinea pig No.	Time elapsed after inoculation.	Amount of serum injected.	Conditions preceding injection of immune serum.	Subsequent course of infection (after injection of immune serum).	Remarks.*
	hrs.	*cc.*			
x194a	1	0.01	No noticeable effects from inoculation.	No symptoms.	No lesions found.
x194b	1	0.1	" " "	" "	" " "
x195a	1	1.0	" " "	" "	" " "
x195b	24	0.01	" " "	" "	Several old hemorrhages in lungs.
x196a	24	0.1	" " "	" "	A few minute foci of old hemorrhages in lungs.
x196b	24	1.0	" " "	" "	No lesions found.
x197a	48	0.01	" " "	" "	Rather diffuse old hemorrhages in lungs.
x197b	48	0.1	" " "	" "	No lesions found.
x198a	48	1.0	" " "	" "	A few old hemorrhagic foci in lungs.
x198b	72	0.01	" " "	" "	Several old hemorrhagic foci in lungs.
x199a	72	0.1	" " "	" "	No lesions found.
x199b	72	1.0	" " "	" "	A few old hemorrhagic spots in lungs.

$x_1$100a	96	0.01	Temperature 104°F. in a.m. but animal not apparently sick; no jaundice. 1st day of disease (4 day incubation period). Serum given in p.m.	Temperature 105.5°F. after injection of serum. Following a.m. 102.5°; 101.5° in p.m. No further symptoms.	A few old hemorrhagic foci in lungs.
$x_1$100b	96	0.1	Temperature 104.5° in a.m.; no other symptoms. Serum given in p.m.	Temperature 104.5° in p.m.; normal next day. Animal well thereafter.	Numerous old hemorrhagic foci in lungs.
$x_1$101a	96	1.0	Animal remained well (temperature 102°) after injection of serum.	No change in temperature (102.5°). Animal remained well throughout experiment.	No lesions found.
$x_1$101b	120	0.01	Had had high temperature (104°, 103°) 2 preceding afternoons. Temperature not high in a.m. when serum was given (101.5°).	Temperature 103.5° in p.m.; animal, however, seemed well. No further symptoms. Temperature returned to normal following day. Survived.	" " " (natural refractoriness suspected).
$x_1$102a	120	0.1	Fever since preceding day (103.5–104°). Temperature in a.m. on serum injection 103°; rose to 104° in p.m.	Temperature next day 103.5° in a.m. and 104.5° in p.m., but on following a.m. 101.5°. Afternoon rise to 104° for 2 more days, then return to normal. Animal survived.	Numerous pale hemorrhagic foci in lungs.
$x_1$102b	120	1.0	Temperature 104° in a.m. and 105.5° in p.m. of day before. 104.5° in a.m. of day serum was given in p.m. Jaundice not distinct.	Temperature remained 104.5° in p.m. 102° next a.m. and 101° in p.m. No further febrile or other symptoms. No jaundice developed. Survived.	Pale hemorrhagic foci in lungs.

* All the guinea pigs surviving were killed on July 4, 1919 (21 days after infection) for examination.

TABLE I—*Continued.*

Guinea pig No.	Time elapsed after inoculation.	Amount of serum injected.	Conditions preceding injection of immune serum.	Subsequent course of infection (after injection of immune serum).	Remarks.*
	days	*cc.*			
$x_1$103a	6	0.01	Fever for 3 preceding days (103.5°, 104°, 104.5°). 102° in a.m. of serum injection. Distinct jaundice; animal very weak, bordering on collapse.	Temperature rose to 104° in p.m. after serum injection. Animal gradually became worse, dying 9 days after infection.	*Autopsy.*—Extreme jaundice, and general hemorrhage, especially in lungs.
$x_1$103b	6	0.1	Fever for 3 preceding days (103°, 104.5°, 104.5°). Temperature 104° in a.m. of injection. Slight trace of jaundice.	Temperature 104.5° in p.m. after injection of serum. Fever disappeared next day. Animal well throughout remainder of experiment. Survived.	Extensive foci of old hemorrhages in lungs.
$x_1$104a	6	1.0	Fever for 3 preceding days (104.5°, 106°, 105°). 103° in a.m. of serum injection. Mild jaundice.	Temperature same p.m. 104.5°, but only 101° next day. Jaundice persisted 3 days longer, then faded. Survived.	Extensive foci of old hemorrhages in lungs.
$x_1$104b	7	0.01	Fever for 2 preceding days (105°, 102.5°). Temperature 104° on day of injection. Jaundice slight.	Temperature 104° after serum injection, but 102° next 3 days, returning slowly to normal. Jaundice disappeared in few days.	Rather numerous old hemorrhagic lesions in lungs.
$x_1$106a	7	0.1	Fever 3 preceding days (106°, 104.5°, 103.5°). Temperature 102.5° in a.m. of serum injection. Distinct jaundice. Serum given in p.m.	Temperature 103.5° same p.m., 102.5° next a.m., 103.5° p.m., 102° next a.m., 101° p.m. Jaundice persisted 3 days, then faded. Survived.	Very marked hemorrhagic lesions in lungs.

$x_1$107a	7	1.0	Fever 3 preceding days (105°, 104.5°, 103°). Temperature 102° in a.m. of serum injection. Marked jaundice. Serum given in p.m.	Temperature 103.5° in p.m.; 101–101.5° on days following. Jaundice increased for 2 days after injection of serum, disappearing 5 days later. Survived.	Hemorrhagic foci in lungs less marked than in preceding animal.
$x_1$107b	7	1.0	High temperature for 4 afternoons preceding (104°, 104°, 103°, 104°), but less in mornings (102.5°, 104°, 102°, 102°). Temperature 102.5° in a.m. of serum injection. Jaundice intense for 2 days. Serum given in p.m.	Temperature 103.5° same p.m.; 100° next a.m. Jaundice and collapse. Died 8 days after inoculation.	*Autopsy.*—Typical lesions.

Controls.

Guinea pig No.	Amount of culture.	Course of infection.	Remarks.*
	cc.		
x192a (control).	0.001	Incubation of 9 days, followed by fever for 4 days (104°, 102°, 105.5°, 103°F.). Jaundice noticed on 3rd day of disease; increased in intensity for 3 days following. Death occurred, with temperature 98°, 13 days after inoculation.	*Autopsy.*—Typical lesions.
x192b (control).	0.01	Incubation of 4 days, followed by period of fever for 5 days. Jaundice developed in 7 days and became extremely intense within 24 hrs., remaining so until death, which occurred on 10th day after inoculation, with temperature of 96.5°.	" " "
x193a (control).	0.1	Incubation period 4 days, followed by fever for 48 hrs. (104.5°, 103.5°), then collapse, with temperature 99° on 8th day. Jaundice appeared on day before death, which occurred 7 days after inoculation.	" " "
x193b (control).	1.0	Temperature 103.5° on 4th day after inoculation. Fluctuated for 48 hrs. longer, but was at no time higher. Jaundice appeared on 6th day, increased in intensity rapidly during next 48 hrs., then receded, having disappeared 4 days later. Animal survived.	Killed. Extensive hemorrhagic lesions in lungs.

serum within a period of 72 hours from the time of inoculation with the culture. All remained well.

At the end of 96 hours most of the animals had a temperature of 103–104.5°F. in the morning. The injections of the serum were made in the afternoon. The temperature remained high after the injection but dropped gradually the following day, and the animals recovered rapidly.

At the end of 5 days most of the animals had had a high fever for 2 days. The serum was given in the afternoon. Two of the three guinea pigs treated began to improve the next day, one had a high temperature for a day longer, but all eventually returned to the normal condition.

At the end of 6 days the animals had begun to show more or less jaundice and had had fever for 3 days. One guinea pig which received 0.01 cc. of the serum died 9 days from the time of infection, while those which received 0.1 and 1 cc. recovered.

Experiments were made at the end of 7 days, when the animals had had fever for about 3 days, and a slight decline had begun in the morning. In some there had been jaundice for 48 hours. The serum was injected in the afternoon. The temperature the following morning was quite high in most animals, and in some it lasted for 2 to 3 days, while in others it went down rapidly. Jaundice increased in intensity in some, but gradually disappeared within several days. All recovered except one, which was near collapse when 1 cc. of serum was injected and which died the next day.

Of the surviving guinea pigs, no lesions were found in the lungs of those treated with the serum within 1 hour; and in each group of three guinea pigs treated 24, 48, 72, or even as late as 96 hours (during the incubation period) there was one animal in which no lesion was present. In others, irrespective of the amount of serum given, there were a certain number of hemorrhagic spots in the lungs, even when treated with the serum within 24 hours. In one instance there was no lung lesion in a guinea pig treated with 0.01 cc. after 5 days. These irregularities are explained by the existence of a considerable variation among individual animals in their susceptibility to *icteroides* infection.

On the whole, the results obtained in this series admit of only one interpretation; namely, that the immune serum, when injected during the period of incubation, prevents further development of the infection, and that when used in the early stage of the disease it is capable of preventing a fatal termination of the infection. On the other hand, when guinea pigs are inoculated with a highly virulent culture, the injection of the serum at a period of the disease when jaundice has existed for some time and the animal is nearing collapse seems to have no benefit. Undoubtedly the virulence of the strain employed for the experiments has considerable bearing upon the efficacy with which the immune serum may be used in the late stage of the disease. The less virulent the culture, the greater are the chances of benefit from the injection of the serum after the clinical symptoms have manifested themselves, as shown by the favorable results obtained in the last series of experiments just recorded. The fact must be emphasized, however, that even with a less virulent strain the serum has no beneficial effect when given to a guinea pig which is in a condition bordering on collapse (fall of temperature, with intense jaundice).

SUMMARY.

The use of a polyvalent immune serum of high potency in the treatment of an experimental infection of guinea pigs with *Leptospira icteroides* was found to be of definite advantage in checking the progress of the infection. When administered during the period of incubation the serum was found capable of completely preventing the development of the disease, although on subsequent examination hemorrhagic lesions of greater or less number and extent were found in the lungs of the guinea pigs which survived. Moreover, the serum modified the course of the disease and when used in the early stages of infection prevented a fatal outcome. Employed at a later stage, however, when jaundice and nephritis had been present for several days and the animal was near collapse, the serum had no perceptible beneficial effect. This was, of course, to be expected in view of the incidence of various pathological phases of this disease—nephritis, hepatitis, and other toxic symptoms in succession. In man the clinical manifestations are more gradual and distinct than in the

guinea pig,[3] yet the yellow fever patient whose temperature is subnormal, and who has reached the stage of hemorrhages from the gums, nose, stomach, and intestines, and of uremia and cholemia, would seem to have little or no chance of deriving benefit from the use of a specific immune serum. This latter assumption would probably hold irrespective of the relation which *Leptospira icteroides* proves to have to the etiology of yellow fever.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547.

THE STERILIZATION OF LIPOVACCINES.

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The proposal to substitute suspensions of bacterial bodies in oil for suspensions in saline solution as a vaccine for use in combating typhoid fever and pneumonia theoretically offers great advantages. Provided the vaccination is safe and efficient, it would be of great importance to be able to get the same result with a single application that has, in the past, been accomplished with three doses of the prophylactic.

The evidence so far at hand would seem to show that the vaccination with oily suspensions causes a formation of antibodies comparable with that afforded by suspensions in water, and thus renders it probable that a similar degree of protection may be given by the lipovaccine. The matter is, however, of sufficient importance to justify repeated examination.

When material of this nature is to be widely distributed its sterility should be assured both by the method of preparation and control tests on the finished product. In this respect the methods proposed by Whitmore and Fennel[1] are not altogether satisfactory. The procedures recommended afford many opportunities for contamination, the oil interferes with the action of antiseptics so that these cannot be depended upon for a final sterilization, and lastly it is far from easy to make satisfactory tests for sterility on the finished product.

Rosenow and Osterberg,[2] recognizing these difficulties, propose to kill the bacteria and any contaminating organisms with a water solution of an antiseptic, to form an emulsion with the oil, and finally

[1] Whitmore, E. R., and Fennel, E. A., *J. Am. Med. Assn.*, 1918, lxx, 902.

[2] Rosenow, E. C., and Osterberg, A. E., *J. Am. Med. Assn.*, 1919, lxxiii, 87.

to remove the water by vacuum distillation at a comparatively low temperature. This appears from their statement to be an efficient method of preparation.

In approaching this matter we have followed another line of thought and wish to record our results at this time as a contribution to the subject without, however, attempting to decide which method may in the end be best.

Loeffler[3] has stated that by the application of dry heat bacteria may be killed without destroying their antigenic properties. The temperature he used was 70°C. and the exposure was prolonged for days or weeks. It is known that glassware and fabrics may be sterilized by exposure to dry heat for a number of hours, the length of exposure varying inversely with the temperature. Thus 130°C. for 3 hours is as efficient as 160°C. for 1 hour. The suspensions of bacteria in oil are essentially dry preparations, and it seemed possible that the finished vaccine could be sterilized at an intermediate temperature without destroying its antigenic qualities. As a matter of fact, our results indicate that this may be accomplished with pneumococcus. We have so far been unsuccessful with *Bacillus typhosus*.

EXPERIMENTAL.

Pneumococcus lipovaccine was prepared according to the method of Whitmore and Fennel.[1] Briefly, the bacteria were grown in glucose broth and separated by centrifugalization after about 18 hours growth. The bacterial mass was dried in an oven at 53°C. over lime. The dry mass was ground in a Pyrex jar with steel balls for a number of hours, a mixture of anhydrous lanolin and cottonseed oil (Wesson) added, and the grinding continued. Finally, sterile cottonseed oil containing 0.25 per cent chloretone was added in such amount that each cubic centimeter of the finished product would contain 2 mg. of the dried bacterial powder. The grinding was then continued for a number of hours, the product put into suitable bottles or ampules and sealed as ready for distribution.

Control tests for sterility revealed the presence of *Bacillus subtilis* and sometimes other bacteria in the dried mass in a majority of

[3] Loeffler, F., *Deutsch. med. Woch.*, 1913, xxxix, 1025.

instances. When the dried bacterial mass was clean the finished product was nearly always sterile. The pneumococcus does not survive the drying process. If the dried mass containing *Bacillus subtilis* is ground in oil containing chloretone this contaminating organism will remain viable for months. With experience in manipulation the amount of material lost because of contamination was reduced, but even with the greatest care it always remained high.

In an attempt to overcome this difficulty we exposed the finished vaccine to a temperature of 130°C. for 3 hours in an electric oven (Freas), and to 120°C. for 12 hours, temperatures which are sufficiently high to sterilize any material. The vaccine, heated and unheated, was administered to healthy mice (0.5 cc. subcutaneously), and after varying periods the resistance of the mice to pneumococcus infection was tested by intraperitoneal injection of doses of culture known to be fatal, or multiples thereof. The results of two experiments are given in Tables I and II.

The experiments show that a considerable degree of protection is afforded to mice by the administration of pneumococcus lipovaccine. In the occasional instances in which the vaccinated mice died it was found that the vaccine had not been absorbed, almost the whole mass injected remaining in the subcutaneous tissues. Protection is exerted against at least ten fatal doses of culture. The mice in the experiments reported had been treated with vaccine 35 and 38 days previously. In other experiments in which the interval was 21, 56, and 110 days, there was no evidence of protection, indicating that the immunity following a single dose of lipovaccine is slow to develop and is transient. The heat treatment did not decrease the antigenic qualities of the vaccine appreciably.

The administration of vaccine in practice as a prophylactic against pneumonia is still in the stage of trial. Since at present the qualities of typhoid vaccine are of much greater interest, we have extended our work to include this phase of the subject.

Typhoid lipovaccine[4] similarly heated and unheated was administered to rabbits intraperitoneally, a single dose of 1 cc. being given. The blood was tested for its agglutinin content at intervals there-

[4] The vaccines used in this experiment were obtained from the U. S. Army Medical School through the courtesy of Major H. J. Nichols.

TABLE I.

Protection Test of Mice Treated with Pneumococcus Lipovaccine.

The vaccinating dose was administered 35 days before the test. The test consisted of inoculation with the indicated quantity of a 24 hour bouillon culture injected intraperitoneally with normal salt solution sufficient to make a total volume of 0.5 cc.

Vaccine.	Culture.		Result.			
	Type.	Dilution.	After 24 hrs.	After 48 hrs.	After 72 hrs.	After 96 hrs.
None	III	1:10,000,000	Sick.	Sick.	Dead.	
	III	1:10,000,000	"	Dead.		
	III	1:100,000,000	"	"		
	III	1:100,000,000	Dead.			
	I	1:10,000,000	Sick.	Sick.	Dead.	
	I	1:10,000,000	Dead.			
	I	1:100,000,000	Sick.	Dead.		
	I	1:100,000,000	"	"		
Unheated	III	1:10,000,000	"	"		
	III	1:10,000,000	"	Sick.		Well.
	III	1:100,000,000	"	"		"
	III	1:100,000,000	Dead.			
	I	1:10,000,000	Sick.	Sick.		Well.
	I	1:10,000,000	Dead.			
	I	1:100,000,000	Sick.	Sick.		Well.
	I	1:100,000,000	Dead.			
Heated to 130°C. for 3 hrs.	III	1:10,000,000	Sick.	Sick.		Well.
	III	1:10,000,000	"	"	Dead.	
	III	1:100,000,000	"	"		Well.
	III	1:100,000,000	"	"		"
	I	1:10,000,000	"	"		"
	I	1:10,000,000	"	"		"
	I	1:100,000,000	"	"		"
	I	1:100,000,000	"	"		"
Heated to 120°C. for 12 hrs.	III	1:10,000,000	"	"		"
	III	1:10,000,000	"	"		"
	III	1:100,000,000	"	"		"
	III	1:100,000,000	"	"		"
	I	1:10,000,000	"	Dead.		
	I	1:10,000,000	"	Sick.		Well.
	I	1:100,000,000	"	"		"
	I	1:100,000,000	"	"		"

TABLE II.

Protection Test of Mice Treated with Pneumococcus Lipovaccine.

The vaccinating dose was given 38 days before the test. The test consisted of inoculation with the indicated quantity of a 24 hour bouillon culture of Type I pneumococcus intraperitoneally. The total volume injected was 0.5 cc.

Vaccine.	Dilution of culture.	Result.	
		After 24 hrs.	After 48 hrs.
None	1:1,000,000	Sick.	Dead.
	1:1,000,000	"	"
	1:1,000,000	Dead.	
	1:1,000,000	Sick.	Dead.
	1:1,000,000	"	"
	1:1,000,000	Dead.	
	1:1,000,000	Sick.	Dead.
	1:1,000,000	Dead.	
Unheated	1:1,000,000	Well.	Well.
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	Sick.	"
	1:1,000,000	"	"
	1:1,000,000	"	Dead.
	1:1,000,000	"	Well.
	1:1,000,000	"	"
Heated to 130°C. for 3 hrs.	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	"
	1:1,000,000	"	Dead.

after. As a control the results were compared with those obtained by the administration of three doses of typhoid vaccine in saline suspension intraperitoneally at 5 day intervals. The results are presented in Table III.

The experiment shows that at least for these particular preparations the lipovaccine is less efficient in single doses than is the saline preparation in three doses. The antigenic qualities of the lipovaccine (typhoid) are almost destroyed by heating to 130°C. for 3 hours.

TABLE III.

Agglutinin Production in Rabbits Treated with Typhoid Vaccines.

Rabbit No.	Interval since last dose.	Vaccine.	Serum dilution. 1:20	1:40	1:100	1:150	1:200	1:500	1:1,000	1:2,000	Remarks.
	days										
1	16	Saline.	++++	++++	++++	++++	++++	++++	+++	++	27 days after first dose.
2	16	"	++++	++++	++++	++++	++++	++++	+++	+	
3	16	"	++++	++++	++++	++++	++++	+++	++	0	
4	16	"	++++	++++	++++	++++	+++	++	+	0	
5	17	Lipovaccine I	++++	++++	++	0					Rabbits tested 12 days later show no increase in agglutinins.
6	17	" I	++++	++++	++++	++++	++	0			
7	17	" I	++++	++++	++++	++++	+++	0			
8	17	" I	++++	++++	++	0					
9	17	" II	+++	++	0						
10	17	" II	0	0	0	0					
11	17	" II	0	0	0	0					
12	17	" II	++++	+++	++	0					
		Lipovaccine heated to 130°C. for 3 hrs.									
13	16	Lipovaccine I	++	0	0						
14	16	" I	+++	+	0						
15	16	" I	+++	++	0						
16	16	" I	0	0	0						
17	16	" II	+++	++	0						
18	16	" II	0	0	0						
19	16	" II	++++	+++	0						
20	16	" II	0	0	0						

SUMMARY.

Pneumococcus lipovaccine confers a definite protection against pneumococcus infection in mice. The protective quality is not destroyed, and apparently is not greatly diminished, by heating to 130°C. for 3 hours or 120°C. for 12 hours.

Typhoid lipovaccine gives rise to the formation of agglutinins in rabbits but to a lesser degree than saline suspensions. The antigenic qualities of the typhoid lipovaccine are greatly injured by heating to 130°C. for 3 hours.

COAGULATION IN EMBRYONIC BLOOD.*

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INTRODUCTION.

In the course of an experimental study of the origin of non-nucleated erythrocytes (Emmel (1)), certain observations were made indicating a slower coagulation time and also other striking differences in the reaction of embryonic blood as compared with that of adult blood. The present work represents a more extended study of this subject with the purpose of ascertaining (1) the facts regarding the coagulation time of embryonic blood; (2) to what extent the factors essential to coagulation are comparable in embryo and adult; and (3) whether any of the conditions found in the embryonic blood may be of possible significance with reference to certain types of abnormal coagulation occurring in postnatal life. With but one exception (Boll (2)) there appear to be no data concerning coagulation in embryonic blood recorded in the literature.

Coagulation Process in Normal Embryonic Blood.

Material and Technique.

The present study of coagulation is largely confined to pig embryos 100 to 270 mm. in length. The material was obtained from the uterus under favorable circumstances within a short interval after the killing of the parent.[1] Only embryos in apparently perfectly normal condition were used, in which the hearts were still beating or would, in any

* Presented at the thirty-fifth session of the American Association of Anatomists, Pittsburgh, April 17–19, 1919.

[1] This material was obtained through the courtesy of Swift and Co.

event, respond to stimulation—a point of considerable importance, since it was found that in the blood from embryos in which the hearts were not beating the coagulation time was materially reduced.

In each instance the umbilical cord was ligated and cut and the embryo removed from the uterus. Part of the anterior thoracic wall was then cut away, the pericardial sac opened, and the heart exposed. The needle (platinum) of a graduated hypodermic syringe was immediately inserted into the left ventricle and care taken to obtain the blood without air bubbles or tissue juices. This blood was then transferred in 0.5 cc. quantities to a series of test-tubes, again with care against the introduction of air.

In general the technique used is in accordance with that of Lee and White (3). Test-tubes of various sizes were tried, but the best results were obtained with tubes having an inside diameter of 9 mm. and a length of about 9 to 10 cm. The glassware and syringes, thoroughly cleaned in chromic acid, were rinsed with isotonic salt solution previous to use in every experiment. The coagulation of the blood was determined by tilting the test-tube at short intervals and noting the formation of a sliding clot or gel, sufficient in the latter case to withstand the complete inversion of the tube. These experiments were made at a room temperature of about 21°C.

Coagulation Time and Character of the Clot.

Table I presents the data for the coagulation time of the blood obtained in eighteen embryos of various sizes. These results are typical of about 50 different experiments and observations. In these data it will be observed that the average normal coagulation time for the blood of the embryos is 23.3 minutes.

This is in marked contrast to the results shown in Table II which were obtained for the adult pig. The blood had been allowed to flow into a clean receptacle as it escaped from the cut carotid and jugular vessels of the animal, and the coagulation time determined for oxalated and non-oxalated blood by the same technique as previously described for the embryo.

On the basis of these data it is evident that the coagulation time for embryonic blood is greatly delayed as compared with that of adult blood, the coagulation time for the embryo being about six to eight times greater than that of the adult. In this connection it may be observed that the coagulation time obtained here for the adult blood may be slightly below normal due to some contact with tissue sur-

faces as the blood gushes from the cut vessels, although this does not seem to be sufficient to affect materially our conclusions.

Certain points are to be noted regarding the process of coagulation as observed in these experiments on embryonic blood. The normal

TABLE I.

Normal Coagulation Time for Embryonic Blood.

Size of embryo.	Volume of blood.	Length of time before first evidence of fibrin was observed.	Length of time for formation of clot.	Size of embryo.	Volume of blood.	Length of time before first evidence of fibrin was observed.	Length of time for formation of clot.
mm.	*cc.*	*min.*	*min.*	*mm.*	*cc.*	*min.*	*min.*
100	0.5	25	30	190	0.5	17	23
120	0.5	19	28	200	0.5	19	22
130	0.5	24	30	210	0.5	12	21
135	0.5	18	30	220	0.5	12	24
140	0.5	15	21	240	0.5	18	21
150	0.5	12	22	240	0.5	17	21
160	0.5	12	20	250	0.5	17	24
170	0.5	12	21	250	0.5	17	21
180	0.5	16	20	270	0.5	12	20
Average						16.3	23.3

TABLE II.

Normal Coagulation Time for Adult Blood.

Non-oxalated blood.		Oxalated blood.*		
Volume of blood.	Coagulation time.	Volume of blood.	$CaCl_2$ 0.5 %.	Coagulation time.
cc.	*min.*	*cc.*	*gtt.*	*min.*
0.5	3	0.5	1	4
0.5	3	0.5	2	4
0.5	4	0.5	3	3
0.5	3	0.5	3	4

* A 1 per cent solution of sodium oxalate was used in the proportion of one part of oxalate to eight parts of blood.

clot never attains a density or firmness comparable with that of adult blood. As a rule, the clot is like a mass of semisolid gel, which at its maximum is usually equal in volume to about two-thirds the total quantity of blood. Since this mass or lump may be made to

move back and forth by tilting the tube, it may be appropriately described as a sliding clot. In contrast to the clot of the adult this clot is seldom of sufficient firmness to permit a complete inversion of the tube. Not infrequently the sliding clot is very small, if not entirely absent.

The first indication of coagulation to be observed consists in the appearance of small particles or clumps of fibrin which are almost invariably deposited at the side of the tube. The formation of a complete gel or clot occurs within an average of about 7 minutes after the first appearance of fibrin, as shown in Table I. Within a short time (30 to 50 minutes) this gel retracts into a small compact mass. If the blood is not too greatly disturbed, the initial deposit of fibrin seems to form a nucleus around which the entire mass of fibrin accumulates during clot retraction. In some instances it appears that the total quantity of fibrin may be deposited in a compact mass without the formation of an intermediate gel stage and this accounts for the occasional absence of a sliding clot.

The data indicate some reduction in coagulation time in the oldest embryos, although the differences apparently are not pronounced within the periods of development to which the present study is confined. In this connection it is to be taken into account that size measurements alone cannot always be taken as an absolute index of the age and degree of embryonic development, since embryos taken from the same uterus may differ by as much as 30 to 40 mm. in length.

Analysis of Possible Factors Involved in the Greater Coagulation Time of Embryonic Blood.

The coagulation of blood is primarily dependent upon the interaction of three factors, prothrombin, calcium, and fibrinogen. Since the coagulation of embryonic blood is, as has just been demonstrated, much slower than that of adult blood, the question arises whether these factors are equally active in the embryo or whether other conditions are to be taken into account. The following analysis has been made with the view of obtaining data toward the solution of this problem.

Blood Platelets.

Platelet Content.—Of the fundamental factors of coagulation, one, the prothrombin, is evidently chiefly a derivative of blood platelets. In many cases of pathological hemorrhage, such as hemophilia and purpura hæmorrhagica, the abnormal variations in coagulation are found to be primarily related to quantitative or qualitative deficiencies in this platelet material. Attention was consequently first directed to the question of the platelet content of the embryonic blood.

From an examination of properly stained preparations of the blood from pig embryos it is obvious that platelets are present in great numbers. Jordan (4) has recently shown that blood platelets are abundant even in 12 mm. pig embryos. A quantitative estimation of these elements based upon numerical data is presented in Table III.

TABLE III.

Platelet Count for Embryonic and Adult Blood.

Size of embryo.	Average No. per c.mm.	Size of embryo.	Average No. per c.mm.
mm.		*mm.*	
25	340,000	120	464,000
40	448,000	140	422,000
45	296,000	270	800,000
95	415,000	Adult.	588,000*

* Derived from a number of counts in which the platelet content varied from 544,000 to 932,000.

In making the platelet count, 3 per cent sodium citrate was used, in accordance with the method described by Ottenberg and Rosenthal (5). The averages are based upon three or more counts in each instance. The platelet counts show a surprising number of platelets in the embryonic blood. With the exception of certain instances, as in the case of the 270 mm. embryo, the platelet count is apparently somewhat less than that of the adult. This difference, however, does not appear sufficient to account for the delay in the coagulation of the embryonic blood. With human blood, averaging about 250,000 platelets per c.mm., pathological hemorrhage does not occur until the platelet count falls below 60,000 (Minot (6)[2]). This represents

[2] Minot (6), p. 1105.

a drop of 75 per cent in man, whereas in no instances in pig embryos between 100 and 270 mm. did the platelet count fall below 27 per cent of the average for the adult.

Coagulation Reaction after the Addition of Platelet Preparations from Adult Blood.—In attempting to determine to what extent blood platelets may be involved in abnormal coagulation of blood, it is evident that a quantitative determination of the platelet content is not necessarily sufficient, as in the case of hemophilia, for example, where the platelets may be present in approximately normal number but still be abnormal in their activity (Lee and Minot (7)[3]). In view of this possibility the following experiments were made in which preparations

TABLE IV.

Effect of the Addition of Adult Platelet Material on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		Size of embryo.	Volume of blood.	Coagulation time.	
		Normal control.	Blood plus 2 drops of platelet suspension.			Normal control.	Blood plus 2 drops of platelet suspension.
mm.	*cc.*	*min.*	*min.*	*mm.*	*cc.*	*min.*	*min.*
100	0.5	30	10	200	0.5	22	10
130	0.5	30	5	220	0.5	24	8
140	0.5	21	7	240	0.5	21	11
160	0.5	20	13	250	0.5	21	12
180	0.5	20	4	270	0.5	20	4
Average						23	8.4

of platelet material from adult pig blood were added to embryonic blood with the results given in Table IV. The suspension of platelet material was prepared in accordance with the technique of Le Sourd and Pagniez (8).

The blood, caught in clean receptacles as it escaped from the vessels of the animal, was immediately transferred to 50 cc. centrifuge tubes and mixed with equal volumes of a solution of 0.4 per cent sodium oxalate in isotonic saline solution, the final mixture of blood with the solution containing 0.2 per cent sodium oxalate. These tubes were then centrifuged at 1,500 revolutions per minute for 10 to 15 minutes and a little more than the upper half of the clear plasma was pipetted off

[3] Lee and Minot (7), p. 80.

and again centrifuged at 1,500 revolutions for 15 minutes longer to throw down any remaining cells. This oxalated plasma was then centrifuged in 15 cc. centrifuge tubes for ½ hour at 3,000 revolutions per minute. The clear supernatant plasma was then decanted, leaving a small sticky grayish white mass of platelet material at the bottom of the tube. A quantity of a solution of 0.2 per cent sodium oxalate in isotonic saline solution was then added to each tube, the platelets were stirred up in this fluid, and the resulting suspensions from four tubes collected into one tube and again centrifuged for 15 minutes at 3,000 revolutions. The supernatant fluid was poured off and the platelets from the entire volume of blood (200 cc.) were extracted with 2 cc. of distilled water. The resultant milky white suspension of platelets was used for experimental purposes within 24 hours after preparation.

These data demonstrate striking results upon the addition of platelet suspensions. The coagulation time of 23 minutes for the normal controls was reduced to 8.4 minutes by the addition of platelet material—a reduction of nearly 75 per cent. The process of coagulation was also attended by the formation of a larger and more homogeneous clot or gel. Not only are these results of interest in that the coagulation time can be materially shortened, but they seemed for the moment to emphasize some abnormal activity of the blood platelets or prothrombin as a factor in the greater coagulation time of embryonic blood. But, as will develop later, it was found that this conclusion could not be maintained.

Examination of these results shows that the effect of the addition of platelet material is by no means constant. In some instances the blood coagulated in 12 to 13 minutes, whereas in others the clot was formed in 4 minutes—a period equivalent to the adult coagulation time. This variation may be due, in part, to qualitative differences in the platelet preparation, for the suspensions differed in opacities, and, as emphasized by Lee and Vincent (9),[4] it is difficult to obtain platelet suspensions of constant strength. Platelet material alone was not sufficient to account for the marked degree of variation in the present results. This raised the question as to whether other factors may be involved in the problem. That these results cannot be due to mere dilution of the blood was shown conclusively by tests with isotonic salt solution and distilled water in which the results were negative.

[4] Lee and Vincent (9), p. 407.

Effect of the Addition of Calcium on the Coagulation of Embryonic Blood.

Attention was next directed to the subject of calcium. Table V presents the results of a series of experiments showing the effect of the addition of two drops of 0.5 per cent calcium chloride to embryonic blood. Here again a reduction of over 50 per cent was obtained. While this was not so pronounced as with platelet material, the results are more uniform and show that the addition of calcium has a decided effect on the coagulation process. These experiments seem

TABLE V.

Effect of the Addition of Calcium on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		Size of embryo.	Volume of blood.	Coagulation time.	
		Normal control.	Blood plus 2 drops of 0.5 % $CaCl_2$.			Normal control.	Blood plus 2 drops of 0.5 % $CaCl_2$.
mm.	*cc.*	*min.*	*min.*	*mm.*	*cc.*	*min.*	*min.*
100	0.5	30	13	180	0.5	20	11
120	0.5	28	10	190	0.5	23	12
130	0.5	30	13	200	0.5	22	11
135	0.5	30	11	210	0.5	21	9
140	0.5	21	11	220	0.5	24	9
150	0.5	22	8	240	0.5	21	12
160	0.5	20	8	250	0.5	21	8
170	0.5	21	9	270	0.5	20	9
Average						23.4	10.3

to show a deficiency of calcium in the embryonic blood, which would seriously interfere with coagulative processes. This conclusion, however, is contradicted by the results obtained in the platelet experiments, which indicate that there must have been a sufficient amount of calcium ions available for coagulation.

From these results the platelet experiments alone would seem to indicate a deficiency on the part of prothrombin rather than calcium, whereas in the calcium experiments the situation appears reversed, and a deficiency in calcium rather than prothrombin is indicated.

Reaction of Embryonic Blood to Tissue Extract.

It will be observed that the platelets are used in the form of a suspension. The question arises whether the effect of the suspension on coagulation may not have been due to elements other than the prothrombin content, for it is to be recognized that in addition to prothrombin, blood platelets contain a second substance, thromboplastin (Bayne-Jones (10)), corresponding in its reaction with the phosphatide designated as cephalin (Howell (11)[5]).

In the preparation of the platelet material used in the present experiments the technique was such that the thromboplastin was still retained in the suspension, and it is therefore possible that this substance constitutes an important factor in the preceding results. To determine whether this was the case, experiments were made testing the effect of tissue extract on coagulation, since it has been shown that this active substance is present in such extract (Howell (11)[5]).

TABLE VI.

Effect of the Addition of Tissue Extracts on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.		
		Normal control.	Blood plus 2 drops of heart extract.	Blood plus 2 drops of cord extract.
mm.	*cc.*	*min.*	*min.*	*min.*
100	0.5	20	4	4
140	0.5	19	4	4
170	0.5	18	3	3.5
200	0.5	13	3	4
230	0.5	18	4	4
250	0.5	15	2	3
Average.....		17.2	3.3	3.7

The extracts were made of the heart and umbilical cord of pig embryos about 200 mm. in size. These tissues were rinsed in water to remove any excess of blood and extracts made in saline solution and water by grinding in a mortar with sand. The mixture was then transferred to a 15 cc. centrifuge tube, centrifuged for 5 minutes, and the supernatant fluid removed. This fluid, or extract, was then used in a series of experiments with the results shown in Table VI.

[5] Howell (11), p. 290.

These results clearly demonstrate that tissue extracts have a decided effect upon the coagulation time. Not only is the time greatly reduced, but it is brought down to the normal time for adult blood; *viz.*, 3 to 4 minutes. It appears obvious, therefore, that in spite of the fact that the embryonic blood normally shows a long coagulation time, nevertheless not only does it contain all the constituent elements essential to coagulation, but these elements will also react under proper conditions in a time equivalent to that of the adult blood.

The results were further verified by blood which had come in contact with cut tissue surfaces by being allowed to run over the cut surfaces of the heart and cord and was found to coagulate in 3 to 4 minutes. It should be stated that in all the experiments with tissue extract not only was the coagulation time thus reduced but the clot was of a much firmer character and permitted complete inversion of the test-tube.

From the standpoint of the results with tissue extract alone it would appear that the delayed coagulation time in embryonic blood must be due to some inhibitory factor. From our knowledge of tissue extracts (Howell (11)[6]) it might be assumed that this factor is solely in the nature of an antithrombin or antiprothrombin. The results obtained with calcium, the addition of which also brings about a reduction in coagulation time, are theoretically inconsistent with the preceding assumption, since this involves the neutralization of antithrombin or antiprothrombin by calcium. These facts indicate that in attempting to determine the nature of the inhibitory factor or factors in embryonic blood, consideration must be given to their possible interaction with calcium as well as with prothrombin.

Calcium Content of Embryonic and Adult Blood.

A chemical analysis of adult and embryonic blood was undertaken to ascertain the calcium content of embryonic blood.[7] The results show that instead of a deficiency there is a pronounced excess of cal-

[6] Howell (11), p. 292.

[7] This work was done by Dr. P. G. Albrecht, of the Department of Physiological Chemistry, who kindly furnished us with data from his unpublished results (Table VII).

cium present in embryonic blood as compared with adult blood (Table VII).

Since on the one hand the results of the preceding experiments upon the effect of the addition of calcium indicate a deficiency in this element, while on the other hand the present data show that calcium is quantitatively present in excess, the suggestion arises that the calcium in embryonic blood is present in some combined form which renders the blood deficient in the free calcium ions essential to coagulation.

TABLE VII.

*Determination of Calcium for Embryonic and Adult Blood.**

Quantity.	Amount of calcium.		Ratio of embryo to adult.
	Embryo (200 mm.).	Adult.	
	mg.	*mg.*	
Total blood 100 gm.	9.5	6.74	7 : 5
Plasma 100 cc.	11.73	8.51	7 : 5

* McCrudden's method as modified by Halverson and Bergeim was used (Halverson, J. O., and Bergeim, O., *J. Biol. Chem.*, 1917, xxxii, 159).

Effect of the Addition of Barium and Magnesium on the Coagulation of Embryonic Blood.

Since the calcium is, therefore, evidently present in embryonic blood in some combined form, it becomes of interest to ascertain whether the addition of other salts, such as barium and magnesium, might liberate some of this calcium and bring about a change in coagulation time. With this end in view experiments were made with the chlorides of barium and magnesium in 0.5 per cent strength (Table VIII).

As a control to determine that these results were due to a liberation of calcium and not to an interaction with some other elements, such as prothrombin for example, the same experiments were repeated on oxalated embryonic blood as shown in Table IX.

The results are significant. Like calcium, barium and magnesium, although somewhat slower in reaction and giving a clot of somewhat smaller volume and looser texture, bring about a reduction in coagulation time of non-oxalated embryonic blood, as shown in Table VIII. These elements, in contrast to calcium, do not react with the oxalated

embryonic blood. On the basis of the specificity of calcium these results apparently permit only one conclusion; namely, that the action of barium and magnesium is due to a liberation of an amount of cal-

TABLE VIII.

Effect of the Addition of Calcium, Barium, and Magnesium on the Coagulation Time of Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.			
		Normal control.	Blood plus 2 drops of 0.5% $CaCl_2$.	Blood plus 2 drops of 0.5% $BaCl_2$.	Blood plus 2 drops of 0.5% $MgCl_2$.
mm.	*cc.*	*min.*	*min.*	*min.*	*min.*
135	0.5	25	11	14	18
180	0.5	15	6	8	8
230	0.5	18	6	7	7
250	0.5	21	8	8	10
270	0.5	15	9	10	11
Average.....		19	8	9.8	10.8

TABLE IX.

Effect of the Addition of Calcium, Barium, and Magnesium on the Coagulation Time of Oxalated Embryonic Blood.

Size of embryo.	Volume of blood.	Coagulation time.			
		Normal control.	Blood plus 3 drops of 0.5% $CaCl_2$.	Blood plus 3 drops of 0.5% $BaCl_2$.	Blood plus 3 drops of 0.5% $MgCl_2$.
mm.	*cc.*	*min.*	*min.*	*min.*	*min.*
170	0.5	No clot.	6	No clot.*	No clot.†
270	0.5	" "	7‡	" " §	" " §

* Two more drops of barium added after ½ hour; no clot formed. Three drops of calcium added after 1 hour; clot formed in 6 minutes.

† Two more drops of magnesium added after ½ hour; no clot formed. Three drops of calcium added after 1 hour; clot formed in 7 minutes.

‡ Two drops of calcium used instead of three.

§ Two drops of calcium added after 25 minutes; clot formed in 5 minutes.

cium sufficient to permit the interaction essential for coagulation, thus furnishing confirmatory evidence for the conclusion that the calcium is present in some combined form.

Certain experiments (Table X) have shown that embryonic blood treated with an amount of oxalate sufficient to prevent coagulation can be made to coagulate upon the addition of tissue extract. On the other hand, in similar experiments with adult blood, in no instances where oxalation was sufficient to prevent completely coagulation could coagulation be initiated by the addition of tissue extract.

TABLE X.

Effect of the Addition of Tissue Extract on the Coagulation Time of Oxalated Blood.

Source of blood.	Volume of blood.	Sodium oxalate.		Tissue extract.	Coagulation time.
	cc.	*per cent*	*gtt.*	*gtt.*	
170 mm. embryo.	0.5				19 min.
	0.5			2	2.5 "
	0.5	0.5	1		No clot in 1 hr.
	0.5	0.5	1	3	9 min.
	0.5	1	1		No clot in 1 hr.
	0.5	1	1	3	" " " 1 "
	0.5	1	2		" " " 1 "
	0.5	1	2	3	" " " 1 "
200 mm. embryo.	0.5				13 min.
	0.5			3	2 "
	0.5	0.5	1		No clot in 1 hr.
	0.5	0.5	1	3	5 min.
	0.5	1	1		No clot in 1 hr.
	0.5	1	1	3	" " " 1 "
Adult.	0.5	0.5	1		3 min.
	0.5	0.5	1	3	3 "
	0.5	1	1		3 "
	0.5	1	1	3	3 "
	0.5	1	2		No clot.
	0.5	1	2	3	" "

Fibrin and Fibrinogen.

Regarding fibrinogen, the third of the factors under consideration as essential to coagulation, the following observations were made. With the other two factors it has been possible to show that in a comparison of embryonic and adult blood of the pig, the calcium and platelet content in the blood of 100 to 270 mm. embryos is more or less

comparable with that of the adult. With fibrin certain differences were observed in the course of the preceding experiments. In the coagulation of embryonic blood it was observed that the clot remaining after complete retraction was somewhat larger in tissue extract experiments than in calcium experiments. In both instances, however, the retracted clots were larger than the corresponding resultant clots for normal embryonic blood.

To compare the relative quantities of fibrin formed in embryo and adult, 0.5 cc. quantities of adult and embryonic blood were defibrinated by stirring with clean glass rods drawn out to narrow points.

TABLE XI.

Weight of Fibrin Obtained in the Coagulation of Embryonic and Adult Blood.

Source of blood.	Volume of blood.	Substance added.	Weight of fibrin.
	cc.	*gtt.*	*mg.*
200 mm. embryo.	0.5		0.1
	0.5	Calcium, 3	0.3
	0.5	Platelets, 3	0.2
	0.5	Tissue extract, 3	0.5
190 mm. embryo.	0.5		0.1
	0.5	Calcium, 3	0.3
	0.5	Platelets, 3	0.1
	0.5	Tissue extract, 3	0.5
Adult.	0.5		4.2
	1.0		8.5

Tests were made with adult blood, normal embryonic blood, and normal embryonic blood to which calcium, platelets, and tissue extract had been added. The fibrin collected on the glass rods was freed from blood by washing in distilled water and kept in 0.5 per cent formaldehyde until weighed. It was then carefully transferred to weighing tubes, dried over night at 55°C., and weighed. Table XI shows the relative quantities of fibrin obtained from embryonic and adult blood.

These results appear to demonstrate clearly a pronounced difference between embryo and adult. In the coagulation of normal embryonic blood only a small amount of fibrin is formed, whereas more fibrin

is formed upon the addition of calcium, and the maximum amount is obtained in the presence of tissue extract. The variation with platelet material is probably due to corresponding variations in the strength of the suspension used. The maximum amount of fibrin formed in embryonic blood is about 12 per cent of that obtained from an equal volume of adult blood. If the quantity of fibrin can be taken as a fairly reliable index of the relative amount of fibrinogen present, it appears that, in contrast to the comparative calcium and platelet content, the fibrinogen content of embryonic blood is far below that of adult blood. This difference in fibrin and fibrinogen content is evidently not primarily responsible for the greater coagulation time of embryonic blood, for in the presence of tissue extract, in spite of the smaller amount of fibrinogen, the coagulation time becomes equivalent to that of the adult (Tables II and VI).[8]

Bile.

The delay in coagulation of embryonic blood is primarily a result of some condition pertaining to calcium. Furthermore, it has also been shown that this is due not to a deficiency in the calcium content, but to some combination of the calcium which renders it unavailable for coagulation.

A consideration of the various types of pathological hemorrhage in man shows that in jaundice or icterus conditions are encountered which are especially significant with reference to the present subject. In jaundice there is a marked delay in the coagulation time of the blood. In the history of the subject this delay has been assigned to

[8] In a microscopic study of the formation of fibrin an observation was made on the behavior of erythrocytes which seems worthy of note. Gütig in a study of adult pig blood states that the erythrocytes never show rouleau formation (Gütig, K., *Arch. mikr. Anat.*, 1907, lxx, 635). In contrast to this, in our own experiments with vaseline-sealed preparations of adult blood, definite and characteristic rouleau formations were observed. It appears much more difficult to demonstrate rouleau formation in the pig than in man, but rouleaux may nevertheless be obtained in great abundance in preparations of pig blood, if care is taken not to use a drop so large as to obliterate the rouleaux or so small as to result in too great pressure of the cover-glass upon the corpuscles. In embryonic blood rouleau formation was not observed even in the oldest embryos.

various causes, but in the more recent literature, as demonstrated by Lee and Vincent (12) and stated by Wells (13), there is a convergence of opinion toward the conclusion that "in icterus a notable tendency to hemorrhage seems to depend upon the binding effect of the calcium of the blood by the bile pigments."[9]

This conclusion becomes highly suggestive with reference to embryonic blood, for it has recently been shown by Bang (14) that in the new-born infant a notable amount of bile pigment is constantly present in blood obtained from the umbilical cord. This pigment shows a constant increase during the first few days, and, if marked, the transition is readily made to the condition designated clinically as icterus neonatorum. Ylppö (15) in his earlier article on new-born and premature infants obtained similar results, but with the exception of the horse, comparable conditions were not found in other mammals. In the wolf, deer, leopard, yak, dog, and pig, in contrast to horse and man, only small quantities of bile pigment, if any, were found in blood of the new-born animal at birth. He concludes that toward the end of the intrauterine life of the horse and man there is a quantity of bile constantly present in the circulating blood, and suggests that the same may be true in the intrauterine life of mammals in general, but in the latter case the excess of bile in the circulation has largely disappeared at the time of birth; or in other words, that with respect to the functional activity of the liver and excretion of bile, the young of the horse and man as compared with other mammals may be regarded as prematurely born.

These results with reference to newly born animals suggest the possibility of the presence of bile in the blood of the pig embryos used in the present experiments and that this bile may be a factor of primary importance affecting the process of coagulation. To determine this point a qualitative examination was made of serum obtained from the blood of 100 to 270 mm. embryos. Tests were made in accordance with Huppert's technique in which the bile pigments are precipitated with milk of lime, freed from the precipitate by boiling in strong acid-alcohol, and their presence demonstrated by the appearance of a typical color reaction. 25 cc. of serum were obtained from

[9] Wells (13), p. 321.

several embryos and the precipitate formed with the milk of lime was treated with 10 to 15 cc. of the acid-alcohol, thus securing the pigment in more concentrated form for the final color test. The results furnished conclusive evidence of the presence of bile in the blood of the pig embryos.

As already indicated, it has been shown that in icterus (Lee and Vincent (12)) the presence of bile may interfere with the participation of calcium in coagulation, and this suggests that the same may be true of embryonic blood. Since the coagulation time for embryonic blood could be greatly reduced by the addition of calcium, and even brought down to the coagulation time obtained for the adult by the addition of tissue extract, as demonstrated in the preceding experiments, it became important to ascertain whether similar experimental conditions could be produced in normal adult blood through the presence of bile.

To this end the following experiments were made in which given concentrations of bile obtained from gall bladders of adult pigs were added to definite quantities of adult pig blood. It was found that the amount and concentration of bile added to the blood is of primary importance in obtaining a given effect on the coagulation time. In order to approximate embryonic conditions, the addition of bile should be such as to bring about a coagulation of the adult blood in about 20 to 30 minutes. To ascertain the requisite amount of bile for this purpose, tests were made with various concentrations (Table XII). The bile for this purpose was removed from the gall bladder, filtered, and then diluted with distilled water to give the percentages of concentration indicated.

On the basis of these experiments, it was determined that three drops of 20 to 25 per cent solution of bile constitute the optimum amount necessary to give a coagulation time of about 20 to 30 minutes for adult blood. The blood when thus treated contains 8 to 10 per cent bile in the final mixture. After the optimum amount of bile necessary for the present purpose had been determined, experiments were made to test the coagulation reaction of adult blood to which bile had been added (Table XIII).

Table XIV gives the weight of fibrin obtained from adult blood treated with bile.

TABLE XII.

Effect of Different Concentrations of Bile on the Coagulation Time of Adult Blood.

Volume of blood.	Bile.		Coagulation time.	Volume of blood.	Bile.		Coagulation time.
	Concentration.	Amount added.			Concentration.	Amount added.	
cc.	*per cent*	*gtt.*	*min.*	*cc.*	*per cent*	*gtt.*	*min.*
0.5	1	2	3	0.5	20	3	27
0.5	3	2	3	0.5	25	1	14
0.5	5	2	3	0.5	25	1	10
0.5	7	2	3	0.5	25	2	22
0.5	10	2	3	0.5	25	3	30
0.5	20	1	3	0.5	25	3	27
0.5	20	2	6	0.5	25	3	25
0.5	20	2	7	0.5	50	1	120
0.5	20	3	30	0.5	100	1	No clot.*
0.5	20	3	20	0.5	100	2	" " *

* Only loose strands of fibrin found.

TABLE XIII.

Coagulation Time of Adult Blood in the Presence of Bile.

Volume of blood.	Coagulation time.					
	Blood plus 3 drops of bile.	Normal control.	Blood plus 3 drops of 0.5% $CaCl_2$.	Blood plus 3 drops of 0.5% $BaCl_2$.	Blood plus 3 drops of 0.5% $MgCl_2$.	Blood plus 3 drops of tissue extract.*
cc.	*per cent*	*min.*	*min.*	*min.*	*min.*	*min.*
0.5	20	27	15			4
0.5	20	20	8			3
0.5	25	25	8	10	10	2
0.5	25	27	12	13	15	3
Average.....		24.75	10.75	11.5	12.5	3

* Extract was made from embryonic heart.

TABLE XIV.

Weight of Fibrin Obtained in the Coagulation of Adult Blood in the Presence of Bile.

Volume of blood.	Amount of 25% bile added.	Other substances added.	Weight of fibrin.
cc.	*gtt.*	*gtt.*	*mg.*
0.5			4.2
0.5	3		1.2
0.5	3	Calcium 0.5%, 4	5.4
0.5	3	Tissue extract, 4	5.0

These results appear significant. The coagulation time of the adult blood when delayed to about 25 minutes by the addition of bile can, by the addition of calcium, barium, and magnesium, be reduced to an average of 10.75, 11.5, and 12.5 minutes, respectively, and upon the addition of tissue extract it can be brought back to the normal time of 3 minutes. Again, whereas in the presence of bile the amount of fibrin formed is reduced to about 30 per cent of the normal, the addition of tissue extract brings the amount back to normal and indeed in the present experiment slightly in excess. These results accord to a remarkable degree with the results previously obtained for the embryo (Tables V, VI, VIII, and XI) and demonstrate that by the mere addition of bile conditions can be produced in the adult blood which appear essentially identical with those of the embryonic blood.

Since in the course of the preceding analysis of the factors involved in coagulation no significant differences could be demonstrated between the blood of the embryo and that of the adult, other than the presence of bile in the former, the data justify the conclusion that it is the bile content found in the circulation of 100 to 270 mm. pig embryos that constitutes the primary factor in the greater coagulation time of their blood.

DISCUSSION.

When, in the earlier phases of this study, it was found that the greater coagulation time of embryonic blood could not be due to a quantitative deficiency in blood platelets as compared with adult blood, the suggestion arose that possibly the condition might be comparable with that of hemophilia, for it seems to be well established that in the latter condition also the platelet content numerically is essentially equivalent to that of normal blood (Lee and Minot (7)[3]). Subsequent data, however, have shown that such a comparison cannot be made. On the contrary, instead of the condition in the embryonic blood being comparable with hemophilia, it has been found to be more nearly that of icterus in which the bile constitutes the primary factor in the abnormal coagulation time.

Since it appears that the inhibitory action of bile is due in a large degree to the combination of the pigment with calcium (Wells (13)), the question arises as to how the process of coagulation is initiated in

normal embryonic blood by the addition of tissue extract. In the calcium experiments the results are due primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. With tissue extract apparently the free calcium ions requisite for the initiation of coagulation must be liberated through some interaction with the constituents of bile. Such an interaction seems clearly demonstrated in the preceding experiments with adult blood to which bile had been added (Table XIII). This is in contrast to the results of Lee and Vincent (12) who, in their study of obstructive jaundice, state that they "attempted to determine whether the action of bile could be neutralized by cytozyme or serozyme, but no results were obtained."[10]

This suggests that the normal coagulation of embryonic blood, as far as bile is concerned, involves a process comparable with that observed after the addition of tissue extract or cephalin, but on a smaller scale. In the embryonic blood *in vitro*, through the disintegration of cellular elements, a certain amount of tissue substance (cephalin (?)) is slowly set free in the plasma. This tissue substance neutralizes the bile and ultimately liberates a sufficient amount of calcium to bring about coagulation.

Of interest in this connection is the fact that the first formation of fibrin takes place almost invariably at the side of the tube (page 180). This fibrin is situated at a level between the red cells and supernatant plasma, probably because of the disintegration of the white cells and platelets which tend to accumulate at this level, an observation confirmatory of that made by Schmidt (16) "that clotting begins always in the white layer." These cellular elements coming in contact with the sides of the tube evidently liberate some substance presumably comparable with cephalin which in turn initiates the formation of fibrin at this location.

The fact that there is a pronounced difference in the quantity of fibrinogen in the blood of the embryo as compared with that of the adult, and that under proper conditions the coagulation time becomes the same for both embryo and adult, demonstrates that the quantity of fibrinogen present in the blood does not play an important part in

[10] Lee and Vincent (12), p. 63.

determining the coagulation time of blood, a conclusion confirmatory of Whipple's observation (17)[11] in certain cases of purpura and hepatic cirrhosis, but in marked contrast to Ottenberg's statement (18) that "in hemorrhagic diseases of the newborn and in chloroform poisoning the prolonged coagulation time is due to a deficiency of fibrinogen."[12]

While the amount of fibrinogen is, therefore, of minor importance as far as coagulation time is concerned, it is to be noted that the actual quantity of fibrin formed in the embryonic blood was greater upon the addition of tissue extract than in the experiments with calcium. In the recent study of the effect of bile on the clotting time of blood, Haessler and Stebbins (19) conclude that bile, through its bile salts, interferes with the conversion of fibrinogen into fibrin. It is consequently possible that the greater amount of fibrin obtained with calcium may be due to its interaction primarily with bile pigments, whereas the action of tissue extract involves not merely the bile pigments but also the bile salts, thus accounting for the increased amount of fibrin. On the other hand, it must be observed that in the experiments with adult blood and bile (Table XIV), upon the addition of calcium an amount of fibrin was obtained essentially equivalent to that obtained upon the addition of tissue extract.

SUMMARY.

1. In pig embryos of 100 to 270 mm. the average coagulation time of the blood was found to be about 23 minutes. This represents a coagulation time six to eight times greater than that obtained for the adult.

2. The first evidence of coagulation in the blood of these embryos consisted in the appearance of small masses of fibrin deposited almost invariably at the side of the test-tube. The ensuing coagulum was, as a rule, in the nature of a sliding clot, never attaining any marked degree of density or firmness.

3. In an analysis of the possible factors involved in this greater coagulation time of embryonic blood, it was found that numerically the blood platelets varied from 415,000 to 800,000 per c.mm., a content not differing in any significant degree from that of the adult, in

[11] Whipple (17), p. 390.

[12] Ottenberg (18), p. 308.

which the average was found to be about 588,000 with a variation from 544,000 to 932,000.

4. The addition of platelet material obtained from adult pig blood reduced the coagulation time for embryonic blood to an average of 8.4 minutes, a decrease of 75 per cent.

5. The addition of two drops of 0.5 per cent calcium chloride reduced the coagulation time for embryonic blood to an average of 10.3 minutes, a reduction of over 50 per cent.

6. The addition of tissue extract to embryonic blood reduced the coagulation time to an average of 3.7 minutes, a time essentially equivalent to that obtained for adult blood. The clot was of a much firmer character than that obtained either in the normal coagulation or in the calcium experiments.

7. Chemical analysis demonstrated a calcium content in embryonic blood in excess of that of the adult, in the proportion of 7 : 5.

8. In view of the results indicated above, it became evident that the calcium in embryonic blood must be present in some combined form. This conclusion is supported by experiments in which barium and magnesium brought about a reduction in coagulation time in non-oxalated embryonic blood, but did not have this effect when added to oxalated blood, indicating in the former case, on the basis of the specificity of calcium, a liberation of free calcium ions. With oxalated blood, it was also found that under certain conditions coagulation could be brought about by the addition of tissue extract.

9. That the fibrinogen content plays no important part in coagulation time is shown by the fact that whereas the maximum amount of fibrin obtained by defibrinating embryonic blood was about 12 per cent of that obtained from the adult, nevertheless upon the addition of tissue extract the coagulation time of embryonic blood becomes equivalent to that of the adult.

10. The presence of bile was demonstrated in the circulating blood of these embryos. Since it was further demonstrated that by the addition of bile to adult blood conditions could be produced essentially identical with those of embryonic blood, and since in the preceding analysis of the factors involved in coagulation no significant differences could be demonstrated between the blood of the embryos and that of the adult other than the presence of bile in the former, the

data justify the conclusion that the bile content in the circulation of 100 to 270 mm. pig embryos constitutes the primary factor accounting for the greater coagulation time in the blood of these embryos. A condition in embryonic blood in some respects comparable with that of icterus is also indicated.

11. In the calcium experiments the results are apparently due primarily to the introduction of calcium ions in excess of the amount which enters into chemical combination with the bile present. With tissue extract it appears that the free calcium ions essential for the initiation of coagulation must be liberated through some interaction with the constituents of bile.

12. The results of this study suggest that the normal coagulation of embryonic blood, as far as bile is concerned, involves a process comparable with that obtained after the addition of tissue extract or cephalin, but on a smaller scale. In the embryonic blood *in vitro*, through the gradual disintegration of cellular elements, a certain amount of tissue substance (cephalin(?)) is slowly set free in the plasma, neutralizing the bile and ultimately liberating a sufficient amount of calcium to bring about coagulation.

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CRESCENTIC BODIES IN ÆSTIVO-AUTUMNAL MALARIA; THEIR MIGRATION AND ATTACHMENT TO THE SURFACE OF THE RED CORPUSCLE.*

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PLATE 13 AND 14.

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Æstivo-Autumnal Parasites.

Since 1879, when malarial parasites were first described, the consensus of opinion has been that the parasites are intracellular, and that each parasite, when young, enters a red corpuscle, staying there until it dies or segmentation takes place, the destruction of the corpuscle corresponding with the segmentation of the parasite. But this is not the case. All malarial parasites are extracellular, that is they are attached to the external surface of the infected corpuscle, and each parasite destroys several red corpuscles. One may trace the destruction of corpuscle after corpuscle by the migrating parasites of æstivo-autumnal infections.

The parasites usually present in the blood of æstivo-autumnal infections are the ring-forms and crescentic bodies. When round bodies are present, they are usually developmental forms of the crescent. The crescent may present an ovoid appearance (1) when bent on itself and viewed from the convex side and (2) when contracted. When the parasite is contracted, the width of the body is increased.

Crescentic Bodies.

The shape of the crescent is similar to that of a caterpillar; the extremities are generally blunt, but one or both may be pointed. That the body of the parasite is wider than it is thick is illustrated by the appearance of one when bent on itself (Figs. 6, 37, and 49).

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Attachment of the Crescent to the Red Corpuscle.—The parasite is extracellular and wraps itself around the corpuscle as a worm wraps itself around a berry (Figs. 1 to 10 and 36). The attachment of these bodies to the external surface of the red corpuscle is demonstrated by the following facts. (*a*) When attached to a corpuscle and seen in profile, the poles of the parasite may be observed extending beyond the periphery of the infected corpuscle in many instances (Figs. 1 to 3, 5 to 8, and 10). (*b*) When viewed from above, one occasionally sees the poles of the attached crescent extending beyond the periphery of the corpuscle (Figs. 9, 12, 26, 30, 32 to 34, and 38 to 40), and in these instances it is often possible to trace the outline of the corpuscle crossing the body of the parasite (Figs. 32, 34, 38, and 39). In rare instances the body proper of the crescent may be seen protruding beyond the periphery of the infected corpuscle (Fig. 48; the dotted line shows where the corpuscle crosses the parasite). (*c*) Occasionally the corpuscle may be seen situated between the body proper and the clubbed end of a crescent bent on itself (Fig. 37).

Ordinarily the corpuscle appears only on one side of the crescent, especially after the corpuscle has been decolorized; but occasionally one may see the corpuscle projecting on either side of the crescent. In these instances the parasites show but little curving and one views them from above (Figs. 13 to 16, 18, 19, 26 to 28, 31, 33, 35, 39, and 40). In the majority the hemoglobin of the corpuscles is more or less intact. Under such circumstances, where the parasite is attached to its full extent, the picture is what one would expect, as the healthy corpuscle is more elastic than the parasite, and spreads out in all directions when pressure is exerted.

Hemoglobin Mounds.

The mounds of hemoglobin substance which the parasite encircles with its cytoplasm for the purpose of attachment and assimilation are easily demonstrated when seen at the periphery of the parasite (at o in Figs. 1 to 3, 5, 6, 29, 32, 35, 47, and 49). These mounds are usually decolorized before the corpuscle itself, but occasionally they may be seen not yet decolorized, in connection with parasites attached to corpuscles showing varying stages of dehemoglobinization, and to corpuscular skeletons (Figs. 41, 43, and 46, at o).

Migration of the Crescentic Bodies.

Crescentic bodies go through migratory stages similar to those of other malarial parasites, and it is possible to find all the stages in one film, especially if the infection is a heavy one and quinine has not been given.

Evidence of Migration.—That the crescentic bodies destroy more than one red corpuscle is strongly suggested by the following facts. (*a*) Heavily pigmented parasites may be found attached to newly invaded red corpuscles. By newly invaded, I mean instances where the hemoglobin appears to be as yet unaltered by the action of the attached parasites (Figs. 1 to 5, 8 to 10, 12, 14 to 20, 22 to 28, and 56). The pigmentation of these parasites is evidence of previous attachments. (*b*) In the same film pigmented parasites are observed on red corpuscles showing varying degrees of dehemoglobinization (Figs. 6, 7, 11, 21, 29 to 40, 47 to 49, and 53). (*c*) Pigmented parasites occur on corpuscular skeletons or remnants of red corpuscles which have been altered by the action of the attached parasites (Figs. 41 to 46). These skeletons may also be seen free from parasites. (*d*) In the same film pigmented parasites are found free from red corpuscles (Figs. 50 to 52, 54, and 55). The pigmentation of these parasites is evidence of previous attachments.

Occasionally a crescent is seen in the act of attaching itself to a fresh red corpuscle before it has conformed itself to the curved surface of the corpuscle (Fig. 56; a careful examination of this figure will show clearly the attachment of the parasite to the corpuscle).

I have never observed the migratory stages in connection with the crescents after the administration of quinine. Possibly the continued use of quinine may inhibit the migration of the crescents without causing their immediate destruction, as they may be present in the blood for some time after quinine has been used without any appreciable anemia resulting. Migration of the crescentic bodies is less frequently observed in the blood than is migration of the young forms of the æstivo-autumnal parasite. This finding might be explained by the fact that the infection is usually recognized before the appearance of the crescents and vigorous treatment instituted. But the large amount of pigment which most of these bodies contain

would indicate that they may do considerable damage before their destructive action is restrained.

A heavily pigmented, full grown parasite attached to a red corpuscle the hemoglobin of which is intact, or nearly so, should suggest that the parasite must have obtained that pigment from another source, and that source was undoubtedly another red corpuscle. It seems to me that this is convincing evidence of parasitic migration.

There is considerable evidence of the migration of the crescentic bodies in the observations of several writers. Free crescents and heavily pigmented crescents attached to healthy appearing red corpuscles have been described and frequently illustrated. Osler[1] pictures free pigmented crescents. Laveran[2] pictures free pigmented crescents and a pigmented parasite attached to a healthy appearing red corpuscle. Mannaberg[3] pictures free crescents and pigmented crescents attached to healthy appearing red corpuscles. Canalis[4] states that the crescents decolorize the red corpuscles and finally become free in the blood, and he illustrates[5] free crescents and pigmented crescents attached to healthy appearing red corpuscles. Thayer and Hewetson,[6] in describing crescents, state that in some instances all trace of the corpuscle may be absent, and Thayer[7] gives figures showing a pigmented crescent on a corpuscle that has not been decolorized, and two free crescents. Manson[8] pictures a pigmented crescent attached to a healthy appearing red corpuscle. Marchiafava and Bignami[9] show a pigmented cres-

[1] Osler, W., quoted from Laveran, A., Paludism, translation by Martin, J. W., London, 1893, 41, Fig. 7, *F* and *H*.

[2] Laveran, A., Paludism, translation by Martin, J. W., London, 1893, 18, Fig. 3.

[3] Mannaberg, J., The malarial parasites. A description based upon observations made by the author and by other observers, translation by Felkin, R. W., London, 1894, Plate 2, Figs. 51 and 52, Plate 4, Figs. 35 to 42, 53, and 54.

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[7] Thayer, W. S., Lectures on the malarial fevers, New York, 1897, Plate 3, Figs. 29, 33, and 41.

[8] Manson, P., Tropical diseases: a manual of the diseases of warm climates, London, Paris, New York, and Melbourne, 2nd edition, 1900, 14, Fig. 9.

[9] Marchiafava, E., and Bignami, A., Malaria, in Stedman, T. L, Twentieth century practice, New York, 1900, xix, Plate 2, Figs. 54, 61, 64, and 65.

cent attached to an apparently healthy red corpuscle, as well as free crescents. Celli[10] pictures a pigmented crescent attached to a healthy appearing red corpuscle and a free crescent. Brumpt[11] pictures a free crescent and Thompson[12] heavily pigmented crescents attached to red corpuscles whose hemoglobin appears to be intact, as well as crescents free from red corpuscles.

EXPLANATION OF PLATES.

PLATE 13.

Magnification, × 1,684.

FIGS. 1 to 5. Heavily pigmented crescents attached to the periphery of healthy appearing red corpuscles, the hemoglobin of which appears to be intact. Hemoglobin mounds encircled by the cytoplasm of the parasites may be seen at o.

FIG. 6. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. Hemoglobin mounds may be seen at o and an attaching filament from the cytoplasm of the parasite may be seen at x. This filament extends beyond the periphery of the infected corpuscle.

FIG. 7. A heavily pigmented crescent attached to a slightly decolorized red corpuscle. The poles of the crescent may be seen extending outside the periphery of the corpuscle.

FIG. 8. A heavily pigmented crescent attached to a healthy appearing red corpuscle, the hemoglobin of which appears to be intact. An attaching filament extending beyond the infected corpuscle is seen at x.

FIG. 9. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed ends of the crescent may be seen bent over the edge of the infected red corpuscle.

FIG. 10. A heavily pigmented parasite attached to a healthy appearing red corpuscle. The clubbed end at the lower part of the picture is seen extending beyond the periphery of the infected corpuscle.

FIGS. 11 and 12. Heavily pigmented parasites attached to fairly healthy appearing red corpuscles.

FIG. 13. Crescentic body attached to a red corpuscle showing Schüffner's granules.

FIGS. 14 to 21. Heavily pigmented parasites attached to healthy appearing red corpuscles.

FIG. 22. A heavily pigmented crescent attached to a healthy appearing red corpuscle. A flagellum taking the nuclear stain may be seen at x.

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[10] Celli, A., Malaria, New York, 1901, 45, Figs. N and R.

[11] Brumpt, É., Paludisme, Précis de parasitologie, Paris, 2nd edition, 1913, Plate 1, Fig. 14.

[12] Thompson, D., The origin and development of gametes (crescents) in malignant tertian malaria: some observations on flagellation, etc., *Ann. Trop. Med. and Parasitol.*, 1914–15, viii, 85, Plate 5, Figs. 12 to 14 and 28 to 32.

FIGS. 23 to 29. Heavily pigmented crescents attached to red corpuscles apparently containing a normal amount of hemoglobin. Fig. 29 shows hemoglobin mounds at o.

FIG. 30. A heavily pigmented crescent attached to a corpuscle partly dehemoglobinized. What is probably an attaching filament, arising from the cytoplasm of the parasite, may be seen extending from x to x.

FIGS. 31 to 35. Heavily pigmented crescents attached to red corpuscles in varying stages of dehemoglobinization. Hemoglobin mounds may be seen at o. In Figs. 32 to 34 the poles of the parasites may be seen to extend beyond the periphery of the corpuscles to which the parasites are attached. In Fig. 33 one of Maurer's rings may be seen in the infected corpuscle at x. In Fig. 35 an attaching filament arising from the cytoplasm of the parasite may be seen at x.

PLATE 14.

Magnification, × 1,684.

FIG. 36. A heavily pigmented crescent wrapped around a decolorized red corpuscle. The external relation of this parasite to the corpuscle is clearly shown.

FIG. 37. A heavily pigmented crescent attached to a decolorized red corpuscle. At x the corpuscle may be seen between the body of the parasite and one of the poles. This parasite could not be within the infected corpuscle.

FIGS. 38 and 39. Heavily pigmented crescents attached to decolorized red corpuscles. The poles of the attached parasites may be seen extending beyond the periphery of the unbroken outline of the infected corpuscles. The outline of the corpuscles may be traced through the bodies of the attached parasites.

FIG. 40. A heavily pigmented crescent attached to a decolorized red corpuscle. At *A* the crescent is bent over the periphery of the infected corpuscle.

FIGS. 41 to 46. Heavily pigmented, healthy appearing crescents attached to corpuscular skeletons. Note the general semilunar appearance of the skeletons. Hemoglobin mounds may be seen at o. Fig. 42 shows the crescent bent on itself. Attaching filaments arising from the cytoplasm of the crescent and attached to the corpuscular skeleton may be seen at x in Fig. 46.

FIG. 47. Pigmented crescents attached to decolorized red corpuscles. Hemoglobin mounds may be seen at o.

FIG. 48. A pigmented crescent attached to a decolorized red corpuscle. The body of the attached crescent extends beyond the periphery of the decolorized corpuscle. The dotted line marks where the corpuscle crosses the parasite.

FIG. 49. A pigmented crescent attached to a decolorized red corpuscle. The crescent is bent on itself, thus giving the appearance of a double bib. A hemoglobin mound may be seen at o.

FIGS. 50 to 53. Flagellated crescents. With the exception of Fig. 53, which is attached to a decolorized corpuscle, these crescents are free from corpuscles. They are healthy appearing and the presence of pigment is evidence of previous attachments. The flagella arise from the chromatin substance of the parasites.

FIGS. 54 and 55. Free pigmented crescents.

FIG. 56. A very heavily pigmented crescent which, I believe, is in the process of attaching itself to a fresh corpuscle, as the red corpuscle shows no evidence of injury to its hemoglobin. A careful examination of this figure will show the hemoglobin of the red corpuscle extending into the substance of the crescent. The large amount of pigment which the parasite contains is evidence of previous attachments.

(Lawson: Æstivo-autumnal malaria.)

(Lawson: Æstivo-autumnal malaria.)

THE HEMIC BASOPHIL.*

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PLATE 15.

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The basophilic or gamma granule of Ehrlich is generally accepted as a characteristic feature of two cell types, the histogenous or tissue basophil, or mast cell, and the basophilic granulocyte of the circulating blood, to which also the term mast cell is commonly applied. Neither of these cells is well understood. Both seem to be characterized in a biological sense by a certain inertia, a certain insensitiveness that manifests itself in a lack of response to irritants capable of arousing quick reaction on the part of cells that seem in general to be of homologous type. In this peculiarity lies, perhaps, part of the explanation for our slight knowledge of the cells in question. The basophil of the blood was, however, placed by Ehrlich among the marrow cell derivatives, since he recognized in it certain similarities to the other granule-bearing cells of the blood and bone marrow. The position thus taken has been opposed by some workers but has nevertheless gained wide acceptance, and the cell is commonly regarded as the complete homologue of the neutrophilic and eosinophilic leucocytes, from which it is believed to differ only in the basophilic staining reaction of its distinguishing granule. The origin and cellular relations as well as the possible function of the tissue mast cell are unknown. Regarded by Ehrlich as an overnourished connective tissue cell, it has been considered by other writers as a clasmatocyte or as derived from one, and again has been looked upon as arising from the lymphocyte or plasma cell. Its possible relation to the leucocytic basophil has been the subject of special debate.

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It has been considered as identical with the latter, but has been more frequently regarded as a separate cell type having nothing in common with the basophil of the blood stream beyond the possession of the same or of a closely similar affinity for certain basic dyes.

Maximow (1910), in his classic studies of the connective tissue elements, concluded that in the lower vertebrates one form may pass over into the other, but in mammals he considered them as entirely separate and distinct except in as far as both are derived from an undifferentiated mesenchymal cell which he calls a lymphocyte or lymphocytoid wandering cell. However, he suggests (1906) that the two forms may be correlated functionally in some way, since the frequency of the respective types varies in different animal species in inverse ratio. Thus the rabbit has few histogenous mast cells in its tissues, but the blood carries a relatively high percentage of basophilic leucocytes, while the contrary condition rules in other species, as in the rat, mouse, and cat. In connection with Maximow's views, it is to be borne in mind that in his opinion the primitive lymphocyte is the common ancestor of all the leucocytes as well as of the defensive cells of the tissues. In a recent communication (1917) he states that lymphocytes grown *in vitro* in a plasma derived from the bone marrow develop into myelocytes.

The socalled oxidase or peroxidase methods for the demonstration of the leucocytic granules supply a new means of approach to the study of the blood cells inasmuch as they provide microchemical reactions whose results appear to depend upon fundamental biological characters of the cellular constituents acted upon by them. Whatever may be the nature of the reacting substance or substances concerned, it is commonly accepted that these methods serve to differentiate the marrow cell from the lymphocytic cell series, and they have become more and more used for this purpose. But it is believed that the differentiation can be pushed a step further so that it becomes possible to subdivide the granulocytes into two groups, the neutrophil and eosinophil showing in common a characteristic brown color reaction toward such a reagent as benzidine, while the basophil exhibits a fundamental difference from these two types through its failure to show such a color change under parallel conditions.

Upon this question of the reaction of the basophilic granule toward oxidase or peroxidase reagents, some difference of opinion is evident in the literature. As far as the tissue mast cell is concerned, Kreibich states that the granules do not react to the Winkler or benzidine solutions. Fischel, on the contrary, obtained a highly selective staining of the granules with a tolidine solution of relatively high acidity. He believed the reaction to be due to a heat-resistant or "pseudo-

peroxidase." Schultze, who first pointed out the practical usefulness of the Winkler method and popularized its use, is quoted by Loele (1912–13) and Pappenheim (1912) as having obtained a negative reaction in the tissue mast cell, but he states in one paper (1909, *a*) that in sections of the skin he obtained a strongly positive result. Unna found that the granules exerted a strong oxidative action on his reagent, the leuco base of methylene blue. He is quoted by Loele (1912–13) and Pappenheim (1912) as having obtained a positive reaction with Winkler's reagent. Loele (1910) obtained only negative reactions with alpha naphthol. He states (1911) that when amino groups are added to the benzene ring, reagents may be obtained that give a black reaction with the basophilic granule, but he considers this non-specific since it is given also by mucus. Personal observation has led to the conclusion that the basophilic granule of the fully differentiated mast cell does not react positively to alpha naphthol or to benzidine, although it is possible that in occasional cells of mast cell type there may be some evidences of a positive reaction in a few of the granules.

The present study has been concerned particularly with the basophil of the blood stream. Some of the writers reporting upon the use of various reagents fail to make specific mention of this cell, thus including it, at least by implication, in the general group of positively reacting forms. In other instances definite statement as to its reaction is made. Schultze (1909, *b*) obtained a positive reaction with the Winkler method and with his own modifications of it, and Kreibich obtained the same result both with the Winkler and with benzidine solutions. Dunn also reports a positive reaction with the Winkler method, but his statement that the reaction is weak contrasts with that of Kreibich, who concluded that the basophils in two cases of myelogenous leucemia studied by him gave the strongest reaction of any of the cell forms present. Pappenheim and Nakano obtained varying results with different reagents. In spite of some indications to the contrary in the tabulated results, they conclude that the reaction is positive and regard the negative reactions observed in some instances merely as evidence of the insensitiveness of the reagent that had been used. Nakano, in a subsequent report of a comparative study of various methods, makes no mention of the hemic basophil in any of the animals studied. He reports a negative reaction on the part of mast cells found in the spleen. It is not clear whether he is speaking here of the blood or of the tissue type, but it is probable that the latter is referred to.

In previous reports of the reactions obtained with an alpha naphthol and with a benzidine method for the demonstration of the leucocytic granules, incidental mention has been made by the author (1916, 1918–19) of the fact that the basophilic granule does not react toward either of these reagents as do the granules of the neutrophil and the eosinophil. The conclusion is supported in a recent paper by McJunkin and Charlton. These statements were made on the basis of the results obtained by the use of reagent solutions made up with 40 per cent alcohol followed by washing of the preparations in water. It has long been known that the basophilic granule is, to a varying degree, water-soluble. This

solubility is said to vary in different animal species and possibly within the same species under varying conditions of health and disease (leucemia (?)). The granule of the cell type found in the blood is more soluble than that of the tissue type. Maximow in particular emphasizes the practical importance of this water solubility as determining the precautions necessary to the successful study of the cell. In his earlier work he followed Michaelis in the avoidance of any exposure of preparations to alcoholic solutions of less than 50 per cent strength but later adopted a strength of 75 per cent alcohol as the least to which the cell could safely be exposed.

In connection with the matter of the water solubility of the gamma granule, it should be pointed out that the authors quoted above have based their conclusions as to the reaction of the cell upon the study of preparations treated with aqueous or with weak alcoholic reagents and washed with water. While such a disregard of the granular solubility would not materially affect the results obtained with the tissue basophil, it would, on the contrary, be expected to interfere more or less seriously with the certain identification of the leucocytic type. Negative findings based upon the use of such reagents must at least be accepted with caution. On the other hand, the usual experience with methods of this sort would indicate that 75 per cent alcohol would not be likely to prove a suitable solvent on account of its probable inhibiting action upon the reacting substance or substances demonstrable by these reagents. Such a solution of benzidine can be used, however, and it seems to give a definite and specific granule staining, although the reaction is somewhat retarded and is best secured through the use of solutions that have been allowed to undergo a certain amount of spontaneous oxidation. Such a solution makes it possible to study the benzidine reactivity of the basophilic granule under conditions calculated to avoid destruction of the granular substance by the staining or washing fluids. Thionin counterstaining gives a striking differentiation of the non-reacting gamma granules as well as distinct nuclear, cytoplasmic, and platelet staining.

Fresh films from a considerable series of cases of myelogenous leucemia were studied.[1] The technique of the benzidine method used

[1] The material necessary for carrying on a study of the hemic basophil as stained by a method founded upon the above considerations was supplied largely by Miss A. L. Gibson, Superintendent of the Huntington Memorial Hospital of Harvard Medical School.

in the staining of these films has been slightly altered from one previously reported, the principal point of difference lying, as indicated, in the avoidance of exposure of the preparations to water or to alcohol of less than 75 per cent strength. 40 per cent alcohol is not entirely destructive to the granules, while 50 per cent alcohol preserves most of them, but their complete preservation can only be assured through the use of the higher concentration.

Method.

The fresh film is fixed in a freshly prepared mixture of one part of neutralized formaldehyde (40 per cent of the gas) with nine parts of 95 per cent alcohol. This fixation is not absolutely necessary, but sharper pictures are obtained with it. The preparation is washed in 75 per cent alcohol and covered in a shallow slide dish with a solution of benzidine in 75 per cent alcohol. The benzidine is made up in 0.5 per cent strength and the solution receives 0.2 per cent of hydrogen peroxide, U. S. P., of tested activity. It is best to allow the benzidine solution to stand at least 24 hours before use in order that a certain amount of spontaneous oxidation may take place, this apparently rendering it more susceptible to further change by the granular substance. Spontaneous oxidation begins in a few hours and is evidenced by a slight brownish coloration of the originally colorless fluid. The solution keeps for weeks. The granular reaction takes place somewhat more slowly than it does when 40 per cent alcohol is used as a solvent and the granules are not so heavily stained, although, as already stated, there does not appear to be any failure in the specific nature of the reaction nor does it appear that the solution fails to stain any granules that would be stained by the 40 per cent reagent. A good granular staining may be evident after 5 minutes, but it has been customary to allow a reaction time of 10 minutes. After being well washed in 75 per cent alcohol the film is stained with thionin. This is made up, as recommended by Maximow (1913), by adding two drops of a 2 per cent aqueous solution of sodium carbonate to 10 cc. of a stock saturated solution of thionin in 75 per cent alcohol. The solution should be allowed to stand 24 hours before being used. It keeps for 2 or 3

weeks. There is no danger of overstaining. 5 minutes has usually been allowed. The film is washed thoroughly in 75 or in 95 per cent alcohol and dried. Balsam neutralized with sodium carbonate has been used for mounting cover-slip preparations, but neutralization is not necessary. The method is applicable to frozen sections.

Results with the Benzidine-Thionin Method.

The method gives a distinct and strongly differential staining of the three granule types of human blood (Fig. 1). Neutrophilic and eosinophilic granules are brown, while, in strong contrast, the basophilic form shows a color varying from a deep opaque purple-red to a more transparent violet-red. This well known metachromatism of the gamma granule is brought out much more sharply by thionin than by the eosinate stains in common use. The color range of the thionin-stained granules is greater in some cases of myelogenous leucemia than in others. Thus in the films from one patient the granules may be almost entirely of the darker staining type, the metachromatism manifesting itself only to a minor degree, while in preparations from another case they may show marked color differences. Here some of the basophils carry granules of one color type, others show only those of the contrasting tint, while again minor variations in the color reaction appear in the individual granules of a single cell. The darker staining granule is apt to be rather smaller than the lighter staining one. It does not usually exceed the alpha granule in size and may be much smaller. The paler type shows a tendency toward occurrence as large round, oval, or irregular, often angulated bodies as large or even much larger than the alpha granule. They may show a central relatively unstained area and in general appear to be swollen and poor in tingible substance. The deeper stained granule appears usually in cells of the smaller diameters (Fig. 2). The deeply stained nucleus of such a cell often suggests that of a lymphocyte, or it may be made out dimly as a lobulated mass simulating, though never reproducing closely, the polymorphous nucleus of a neutrophilic leucocyte. It exhibits in general a tendency toward the formation of the bizarre forms illustrated by Weidenreich. The larger granule, on the other hand, is found in

cells of larger diameter with more faintly stained nucleus, which may be multilobulated but is again different, particularly in its lesser chromatin content, from the type usually connoted by the adjective polymorphous (Fig. 3). The granules are apt to be less numerous and more widely spaced in the cytoplasmic body than they are with the smaller type.

Possible Causes for the Variations in Staining of the Basophilic Granules.

The idea occurs at once that these variations might simply be the result of a greater or less flattening out of the cell by the varying pressure to which it may have been subjected as the film was made. It is unquestionable that a certain amount of variation can be produced in this way, as can be shown in any blood film by whatever method it is made or stained, but it is not believed that the varying appearances described can be due to artifact to more than a minor degree. Basophilic cells of sharply contrasting appearance may be found side by side in thin fields with widely spaced cells, while on the other hand thick fields of closely crowded cells still allow a differentiation of the two general types.

Another question suggesting itself is whether the thionin coloration of the granules may not merely be superimposed upon the brown of a primary though possibly weakened benzidine reaction. With this possibility in mind, films were studied before and after thionin counterstaining and the findings in individual cells at the two stages compared. Cover-slip preparations were divided into sets of small squares by lines drawn upon the reverse side with marking ink, the films were treated with the benzidine solution, mounted in 75 per cent alcohol, and the various fields plotted. The preparations were then counterstained and the cells as finally stained compared with the provisional classification of the first charting. In the preparations thus studied a sufficient number of basophils was encountered to establish the conclusion that the basophilic granular material showed no evidence of a brown coloration after benzidine. In the later preparations the basophils could be picked out with some degree of confidence in the first plotting as colorless bodies with indistinct nuclear outlines and scattered vacuole-like markings

slightly if at all more refractive than the cytoplasmic substance in general. Indeed, some of the granules are evidently overlooked under these conditions, since cells in which, during primary plotting, only a few scattered granules could be made out might present in the final picture a cytoplasmic body crowded with the characteristic basic stained bodies. A parallel plan was followed for the tissue basophil and here again no benzidine coloration could be made out in the fully differentiated cells.

Variation of Granule Types in the Individual Cell.

It is well known that the myelocytes of normal bone marrow as well as those found in the circulating blood in myelogenous leucemia may show a mixed granulation; that is, the presence within a single cell of granules exhibiting contrasting staining affinities. In man, the associated forms are almost always eosinophilic and basophilic, although the coexistence of neutrophilic and basophilic types has been recorded (Bloch and Hirschfeld, Engel). In the guinea pig and rabbit basophilic granules may also occur in association with the pseudo-eosinophilic or beta type. This heterochromatic granulation, as it was termed by Levaditi (1902), has been the subject of considerable discussion, the interest centering in the question as to whether the basophilic bodies are to be considered as actual gamma granules or as neutrophilic, eosinophilic, or pseudo-eosinophilic forms with a peculiar variation in their staining affinity dependent upon incomplete differentiation or upon degenerative changes in the proper granules of the cell. The early discussion was complicated by a confusion of terms, and it was not always clear whether a writer used the word basophilia in its general sense or in its restricted meaning as applied to the peculiar metachromatic basophilia of the true gamma granule (Arnold).

Myelocytes containing eosinophilic and metachromatic basophilic granules are not uncommon in some cases of myelogenous leucemia. They seem to occur particularly in cases showing the wider variations in the appearance of the true basophils. The two granular types occur in varying proportions in different cells and the containing cells vary considerably in size. The larger examples usually contain a pre-

dominant number of eosinophilic granules (Fig. 4), and one gains the impression that as the relative number of gamma granules increases the cell becomes smaller. Thus the smallest cells encountered contain numerous gamma granules with only an occasional alpha form embedded in the compressed field of closely crowded basophilic masses (Fig. 5). The nucleus is most often of myelocytic type, definitely so in the larger forms, but as the cell becomes smaller the nucleus likewise diminishes in size and takes on a heavily stained, shrunken appearance with outlines often masked by the apposed or overlying granules. Where most clearly made out it often shows an irregular outline with shallow indentations or small bud-like protrusions of its substance. Occasionally it may suggest the polymorphous configuration, but the internal nuclear structure is not that of the neutrophilic cell. The appearance of neutrophilic and basophilic granules in the same cell is certainly very rare, but at least one cell has been observed that seems unquestionably to be a polymorphonuclear neutrophil containing a few typically metachromatic basic staining granule masses. Its proper neutrophilic granules have partially disappeared (Fig. 6). In the eosinophilic-basophilic cells the individual granules are usually of pure type, that is, they are definitely brown-stained or thionin-stained, but occasionally cells are encountered in which the individual granules may show a mixed staining reaction. In one cell, as illustrated in Fig. 7, rupture of the cell membrane has allowed a wide dispersal of the granules so that each may be closely examined. It will be seen that granules of three types appear. There are granules of unquestioned alpha type though of variable size and variable depth of color; others that again show variations in size and staining reaction are of undoubted gamma type; while in a third type an underlying brown color appears to be more or less replaced by a metachromatic thionin staining. Here the basic staining substance appears as a scarcely perceptible peripheral darkening of the body in a part or the whole of its circumference or as a definitely reacting area or sector showing variable metachromatic tints and involving little or much of the granular mass. It looks as though the basic staining substance makes its appearance at some focus in the granule and extends thence to involve the whole substance. The purely basic stained granules vary

from minute fragments to large irregular bodies and show a variable staining reaction. The same changes are present in the granules of the cell represented in Fig. 8, but they are not so plainly made out and the cell appears not to be in so good a state of preservation.

Distinguishing Characteristics of the Basophilic Granule.

In correspondence with its failure to react toward benzidine, the basophilic granule differs in other respects from the neutrophilic and eosinophilic types. Its characteristic water solubility has already been mentioned. This may be only an exaggeration of a property present to some degree in the other two forms, but it is still sufficiently peculiar to constitute a distinct type characteristic. It is also resistant to drying. The benzidine-active granules begin to show a gradual loss of their property of benzidine coloration within a comparatively short period after the film is made. The neutrophilic granule shows this change first and most strikingly, but the alpha form also loses its reactivity after a longer period. In films of normal blood 6 months old the neutrophils appear entirely devoid of granules under the benzidine-thionin stain, while the eosinophilic granules are colorless or at the most show but doubtful traces of a brownish tone. In the occasional basophils found in such a preparation, however, the gamma granules show a staining reaction slightly if at all altered from that characteristic of the fresh film. Like results are seen in heated preparations. When fresh films are subjected to dry heat of a temperature averaging 150°C. the benzidine reaction of the neutrophilic granule is quickly destroyed. After 10 minutes no trace of the neutrophilic granules can be seen in benzidine preparations, while the eosinophilic granules react rather feebly. After 20 minutes the eosinophilic granules also appear inert. Meanwhile the basophilic granules have shown no change beyond a possible slight lowering of their color tone, and even after 30 minutes at the given temperature these cells have a natural appearance, although the granules show a lowered color tone and an added emphasis of the central comparatively unstained portion often seen in fresh preparations.

Resistance is shown also to treatment with acids and alkalies. Applied to an unfixed or even to a fixed blood film, a 1 per cent solution of potassium hydroxide in 75 per cent alcohol destroys the neutrophilic granules practically at once so that no trace of them can be found upon subsequent benzidine staining. The benzidine reactivity of the eosinophilic granule is also destroyed, although the granule mass itself can still be made out as a colorless body exhibiting the characteristic refractivity. The gamma granule resists much longer exposure. The same resistance is evidenced toward acid solutions. Short exposure to a 1 per cent solution of acetic or lactic acid in 75 per cent alcohol changes or destroys the reactivity of the neutrophilic and eosinophilic granules without changing the appearance of the basophilic type. Acid and alkaline solutions show the same action when applied to frozen sections of formaldehyde-fixed tissue containing numerous basophils of the tissue type in association with polymorphonuclear neutrophils.

It is well known that variations in the hydrogen ion concentration of the media may affect the granular staining brought about by the so called peroxidase reagents. These variations may alter the color of the stained granules or they may result in total failure of the reaction. The findings in blood films exposed for varying intervals to weak acid solutions suggest that the result is due to a progressive change in the granular substance, and there is a certain amount of evidence that at some stage in the course of its disintegration the granule may become basophilic and inactive toward benzidine. When fresh films are exposed to the acid solution for 30 seconds, washed in 75 per cent alcohol, and stained with benzidine and the stock non-alkalinized thionin, the neutrophilic granules show a variable color reaction. In fresh films the neutrophils are usually filled with a closely crowded mass of opaque greenish blue granules. In preparations 2 or 3 days old the changes are generally more extensive. Many of the granules fail to stain, while the forms that are still demonstrable show wide variation in staining reaction. A few may still show a slight brownish coloration, many are of a dull greenish blue, while scattered through the cytoplasm there appear occasional bodies of about the size or of slightly larger size than the neutrophilic granule which exhibit a metachro-

matic thionin coloration not to be distinguished from that of the granules in a typical basophil (Fig. 9). From their arrangement and general appearance it seems clear that these represent chemically altered neutrophilic granules. The appearance of these cells recalls that of the neutrophil (Fig. 6) found in the blood from a patient suffering with leucemia. Experimental work founded upon this observation is in progress.

DISCUSSION.

The basophilic granule exhibits certain physical and chemical reactions that appear to be different from those characterizing the neutrophilic and eosinophilic types. As compared with them it seems chemically more stable while biologically inert. Study of the cell forms found in the leucemic blood of man suggests strongly that the metachromatic basic staining granular substance arises in some way as the result of changes taking place in the granules of what may be termed the essential or true leucocytes, or myelocytes. The progress of the changes leading to its appearance may be followed in the cells showing the heterochromatic granulation above described. In such cells as the ruptured eosinophilic myelocyte illustrated, the benzidine-active substance appears to undergo a gradual change into a substance no longer endowed with this property of reacting in characteristic manner to a benzidine solution. This change is taken to imply a fundamental alteration in the nature of the granular material. The concomitant acquisition of a basic staining property of peculiar type serves as further evidence of the chemical alteration in progress. In the cells as a whole there occur all stages of the conversion of an eosinophilic cell filled with active granules into cells whose granules appear entirely inert toward benzidine. These inactive granules are water-soluble but resistant to physical and chemical agents destructive to the benzidine-active granular substance. Accompanying the granular changes there appears to be a progressive degenerative change in the nucleus. It shrinks, becomes more compact and more heavily stained, and takes on a crumpled or shrivelled appearance. The end-product becomes a small cell with a gnarled nuclear mass and a thin cytoplasmic envelope crowded

with the basic staining remains of its original granules. In other instances it may be that the degenerative change goes forward less rapidly, or, in other words, the cell is able to maintain a metabolism more nearly equal to the normal so that despite the progress of the granular changes the cell as a whole secures a better approximation to the normal evolutionary course. Under these conditions the nuclear appearance may simulate more or less closely that of the true leucocyte, while there may remain a considerable amount of cytoplasmic substance whose granules tend to be relatively fewer than in the first case and more lightly stained. This is the type usually found in normal blood, while the smaller type is more typical of leucemic blood.

Whether or not the basophil is a true granulocyte has been questioned repeatedly. Harris while studying the tissue mast cell was impressed by the fact that its granules react toward dyes just as mucus does, and after trial of various stains, including the special methods of Mayer, he concluded that the granular material actually consists of mucin, whence he called the cell a "mucinoblast." He describes cells with similar granules which he had observed in the lymphatics of inflamed tissues and in the blood and believed that these forms arise from the "hyalin" cells. A few years later Pappenheim expressed his belief in the mucinous nature of the basophilic granule and the view has been urged repeatedly by him and his followers. Like Weidenreich, he concluded that the mast cell may represent a degenerated lymphocyte, but while the latter derived the basophilic granules from particles of nuclear material, Pappenheim regarded them as the product of a mucoid degeneration of the spongioplasm, and from a further study of their staining reactions arrived at the conclusion that they are in fact made up of an alkaline mucolipoid (Pappenheim, 1906, 1912). Since the early work of Ehrlich it has been generally accepted that the myelocytic granules show in the first stages of their development a basophilic staining reaction. It is usually accepted that this basophilia is not of the metachromatic type characteristic of the true gamma granule. Pappenheim, however, regards the basophilic granules of normal blood and bone marrow as immature examples of the alpha or of the special or beta granules. He considers that the degenerated lymphocytic type of the cell occurs in the blood of man only in leucemia. For Pappenheim and his followers, therefore, three possibilities are given as to the significance of the basophilic granules as they occur in the cells of the blood and bone marrow of man and other vertebrates: they may arise through degenerative changes in a non-granular lymphocyte (such a cell does not, it must be remembered, represent, in the so called monophyletic view of this school, a series distinct from the granule-bearing cells); they may represent an early developmental stage of the alpha or beta granules, or "an aplasia, abortive formation, or degeneration of the

'ripe' granules;" and, lastly, "there may be a combination of both the above possibilities in that the granules appear in such lymphoid cells as were originally destined to the formation of oxyphilic granule cells" (Pappenheim and Szécsi).

The contention that the basic staining granules occurring in the cells of mixed granule type are early undifferentiated forms of one or another granular variety would not seem to apply to the preparations described in the present paper, for it is well known that the various peroxidase methods are capable of demonstrating a positive granular reaction in cells of the myeloblast stage, where the usual stains fail to show any evidence of granule formation. All the evidence at hand indicates that benzidine is fully sensitive enough to accomplish this result. It is, therefore, hardly possible that definitely formed granules of essential type could fail to give a positive benzidine reaction.

Weidenreich attacked the whole plan of leucocytic classification based upon staining reactions instead of upon morphological considerations and objected in particular to the prevailing conception of the hemic basophil as a normal leucocyte. He considered this cell as different chemically and physiologically from the other leucocytes in all animals except the guinea pig. In the latter he recognized a true mast leucocyte with specific granules and a regular course of development from a mast myelocyte of the bone marrow. In all animals other than the guinea pig he regarded the basophil as a degenerated lymphocyte whose granules were derived, directly or indirectly, from particles of nuclear material that had become broken off and extruded into the cytoplasm. In support of this view he urged his failure to find a centrosome in any basophils except those of the guinea pig, and the further argument that the morphological peculiarities of the nucleus are not those of the true leucocyte but rather those of a degenerating nucleus of lymphocytic type. Downey (1912) believes that the tissue mast cell in the cat is developed from the lymphocyte and that its granules are derived from particles of nuclear material. Blumenthal concluded that the basophilic leucocyte is derived from the same finely granular basophilic myelocyte that gives rise to the neutrophilic and eosinophilic series, the last two representing the normal lines of development, while the basophil is a pathological form appearing in numbers only under conditions of disease. Gulland suggested that the tissue mast cells are leucocytes specially differentiated from long residence in the connective tissues and that the basophils of the blood and bone marrow may be examples of such connective tissue cells which have escaped into the blood stream.

Functionally, the basophil seems to be inactive. It is encountered only infrequently in normal blood, and the laws governing its increase in the blood or tissues

under pathological conditions do not appear to be those obeyed by the other blood cells. Fahr concluded that it is not phagocytic and that it appears to take no part in the ordinary phenomena of leucocytosis, although he concluded, on the basis of its disappearance from the peritoneal cavity after the injection of virulent organisms, that it may exhibit a negative chemotaxis. He showed that a negative chemotaxis common to all the leucocytic forms may become positive after immunization in all types except the basophilic. Routine clinical and laboratory experience would seem to offer little in favor of the investment of the cell with properties of phagocytosis or other defensive activity.

In studying septic and aseptic inflammations in animal tissues Maximow (1904, 1905) failed to find any evidence of phagocytic activity on the part of the basophilic leucocyte, nor was there any clear indication of a direct response on its part to bacterial invasion, although the cells are present in numbers in the marginal tissues of a purulent focus. He believed that both basophilic forms give off their granular material into the surrounding tissues either by simple extrusion of the granules or as the result of a complete disintegration of the cell. This disintegration of the mast cell in an inflammatory area may be extensive, and the granular material may be taken up by the phagocytic leucocytes or by polyblasts which attack the degenerating cells, or it may be deposited, directly or indirectly, in fixed connective tissue cells. He describes polyblasts showing a generalized metachromatic staining of cytoplasm and nucleus, this appearance being accompanied by obvious degenerative changes in the cells. In the scar tissue resulting from an inflammatory process the mast cells still appear, sometimes in considerable numbers. He is inclined to regard the granular material as a nutritive substance that may be concerned in some way with tissue metabolism or with defense processes. Greggio likewise believes that the tissue mast cell may be a sort of unicellular gland capable of existing in the tissues in an undifferentiated resting stage until aroused by local nutritional disturbances such as occur, for example, in inflammation, when it may become active and take up its specific function of elaborating granular material and discharging it into the surrounding tissues. Harris and others have considered it possible that the granular material thus discharged may be bactericidal, but there is no evidence to support this view.

The conception of the gamma granule as the product of degenerative changes is in harmony with observations recorded in the literature as well as with certain facts of common experience. Maximow (1905) describes the appearance in old pus collections of a granular material having the tinctorial characters of the metachromatic granules, and, more significantly, notes the presence of metachromatic granules in the cytoplasm of large actively phagocytic cells scattered about an abscess cavity in rabbit tissues. He is unable to explain their appearance here but considers it possible that they may be a synthetic cellular product derived primarily from some constituent of pus. It would be, perhaps, no less reasonable to regard them as the product of the disintegration of cellular material that is undergoing digestion within the phagocytes. The disintegrating

mast cells described as occurring in the margins of an abscess and the metachromatic change accompanying the obvious breaking up of the polyblasts may likewise be interpreted as evidences of the degenerative changes that are taking place in and about such areas. In this connection it is of interest that Harris describes a "smooth muscle mast cell" as occurring in the muscular wall of a tubercular intestine and in the vascular media in tumors. Here obvious degenerative changes in smooth muscle cells were accompanied by the appearance in the cell cytoplasm of numerous small metachromatic basic stained granules. Wolff found that after the injection of spermatozoa into the peritoneal cavity of the guinea pig the phagocytic mononuclear cells closely resembled mast cells, their cytoplasm being filled with granules regular in size and arrangement and exhibiting a definite metachromatic staining reaction to methylene blue although not to thionin or to cresyl violet. He showed that the granules represented bits of the disintegrated spermatozoa. These findings suggest that the property of reacting metachromatically toward certain basic dyes may be a general property of disintegrating cellular material. Incidentally, they emphasize also the weakness inherent in any method of granular classification founded merely upon staining reactions. The objection may apply to the concept set forth in this paper, but despite our ignorance as to the final significance of the granular reaction provoked by the so called peroxidase reagents, it is believed that the conclusions drawn have in their favor demonstrable granular differences that are dependent upon variations in the granular substance more fundamental in their nature than any that can be made evident through the single factor of a varying reaction towards the aniline dyes.

Occasional attempts have been made to produce experimental basophilia or basophilic leucocytosis. Levaditi (1902) reports basophil counts of 10 to 13 per cent in rabbits and guinea pigs following the subcutaneous injection of bacterial toxins and the intraperitoneal injection of a specific hemolysin. It is noteworthy that the basophil increase shown in his charts occurs comparatively late, and the question is suggested whether this increase may not constitute a function of degenerative rather than of regenerative or proliferative changes in the blood cells. Such an explanation may apply also to the few other instances of experimental basophilic leucocytosis that have been reported. Thus, Pröscher (1904), in experiments carried out with Pappenheim, claims a basophilic leucocytosis in the rabbit as the result of the injection of a hemolytic poison, "phrynolysin," and several years later (1909) he again observed it in the rabbit following the injection of supposed cultures of an ultramicroscopic smallpox virus. Pappenheim and Szécsi obtained a basophilic leucocytosis in the rabbit with saponin or saponin combinations, and they quote Kasarinoff as having found it in the bird after saponin injections. Two facts are of interest in these reports. First, the agents found capable of producing an experimental increase of the basophils are usually substances having a direct destructive action upon the blood and blood-forming organs, and, second, it is noticeable that with the increase in the basophils there occurs a rise in the numbers of the eosinophils or pseudo-eosino-

phils. Thus, in two rabbits that had received the smallpox virus the basophils increased to counts of 18 and 16 per cent (the stated normal is 4 to 8 per cent), while the polymorphonuclear eosinophils rose to 4 and 3½ per cent respectively (the normal is given as 0 to 0.57 per cent), and pseudo-eosinophilic myelocytes made their appearance in the circulating blood, rising to levels of 7 and slightly over 7 per cent. In a recent report dealing with experimental benzol poisoning in rabbits, Weiskotten and Steensland record the occurrence in animals of a definite basophil increase. This appears comparatively late and may conceivably be the expression of a degenerative process set up in the blood-forming tissues.

The most striking instances of experimental basophilic leucocytosis in the literature were obtained by Schlecht in guinea pigs inoculated intraperitoneally with foreign sera. In animals dying after repeated intraperitoneal injections of horse or sheep serum he claims to have observed marked increase in the basophils, these cells reaching counts of even 25 to 28 per cent of the total leucocytes. He was interested primarily in the study of eosinophil variations and believed that he could find some evidence of a reciprocal variation in the numbers of eosinophilic and basophilic cells, the two types varying in inverse ratio. His animals showed at first a decrease of the eosinophils and basophils; this was followed by a fluctuating rise in the eosinophils with a variable but distinct basophilic increase. During the last 3 or 4 days before death occurred the eosinophils showed a marked drop in their numbers and, in one animal, complete disappearance, while the basophils rose more or less abruptly to the unusual levels quoted. The cause of death in these animals is not made clear.

There is on record, then, a certain amount of experimental evidence that an increase in the eosinophils or pseudo-eosinophils of animals may be accompanied by fluctuations in the number of basophils. If the suggested relation between the cell types holds it must, however, depend upon some factor not always operative in conditions capable of exciting an increase in the eosinophils of the circulating blood, since there is but little evidence in its favor in the eosinophilias commonly encountered in man aside from the eosinophilia occurring in leucemia. In three cases of trichinosis, of which two had eosinophil counts of 23 and 26 per cent, while the third, a moribund patient, presented a terminal drop in the count to 1 per cent, no increase in the basophils was found. No increase was seen in two cases of bronchial asthma recently under observation, both of which presented slight eosinophilia. Slightly more suggestive are the findings in a rabbit suffering from a well developed case of aural mange (*Dermatodectes cuniculi*). Here a count of 1,000 cells showed 4.6 per cent

eosinophils and 6.6 per cent basophils. The latter figure can hardly be considered as constituting a distinct basophilia, since the normal range of the cell in this animal is usually accepted as from 2 to 5 per cent, but at least it is a high normal. One case of parasitism in man has been seen in which there appears to have been a coincident rise in eosinophils and basophils. The case was one of hookworm infection and the differential count (500 cells) showed 13.8 per cent eosinophils and 4.6 per cent basophils. The eosinophilia obtained by Herrick in guinea pigs treated with *Ascaris* extracts was accompanied by fluctuations in the basophil count. The highest level recorded is 7.5 per cent.

The question as to the nature of the hemic basophil is, of course, bound up intimately with that as to the existence in the bone marrow of the ancestral forms of the cell. Consistently with their denial of the existence of a true basophilic leucocyte, the followers of Pappenheim fail to find a true basophilic myelocyte in the bone marrow (Benacchio, Kardos, Pappenheim (1912)). Weidenreich likewise denies its existence except in the guinea pig. Maximow (1913), on the contrary, finds both forms in various animal species. Both are, however, more infrequent than cells with eosinophilic or neutrophilic granules and in the bone marrow the leucocytic phase predominates numerically over the myelocytic. Maximow's findings are supported by Downey (1914) and by Ringoen for the rabbit and guinea pig. It is to be noted, however, that the basophil is stated to exhibit a heteroplastic type of development as contrasted with the homoplastic type characteristic of neutrophilic and eosinophilic cells. While the last two multiply almost always through the mitotic division of cells already possessing granules, the basophilic leucocyte is developed characteristically without mitosis from a non-granular cell through the gradual appearance of the basophilic granules in its cytoplasm and subsequent nuclear changes toward the usual leucocytic type. These changes are not considered as degenerative. Downey describes them as occurring in the eosinophil series also.

Realizing the difficulties imposed in the way of a full acceptance of his views by the doctrine of a purely heteroplastic genesis of the basophilic leucocyte, Maximow made the point the subject of study in a number of animal species. He finds (1913) dividing mast myelocytes in the guinea pig, rabbit, rat, and rarely in man. He admits that it requires long search to demonstrate an example in human bone marrow, and it may perhaps be said that the figure reproduced as an example of a dividing cell is not particularly convincing. On the other hand, they are said to be frequent in the guinea pig. It will be recalled that Weidenreich came to the conclusion that this is the only animal species of several examined in which the existence of a true basophilic myelocyte with derivative leucocytes could be proved. In this connection it is further of interest that

Levaditi (1902) described the "ovoid granule" of the guinea pig as a distinct type that is peculiar to this animal and found in no other. He finds evidence for this view in its greater resistance to solvents (Maximow also considers it relatively insoluble in water) and in the fact that it is stained by the tri-acid mixture, which fails to stain the basophilic granule encountered in other species. Under benzidine-thionin the basophilic cell of guinea pig blood sometimes suggests the cell of mixed granule type found in the leucemic blood of man rather than the true basophil of human blood. A few of the granules may take a dull brown color and the thionin tints of at least a portion of the others may seem to be combined more or less obviously with a brown coloration as though superimposed upon it. It is possible that the basophil of the guinea pig possesses granules that are relatively better preserved than those of other species, and in this fact may lie an explanation for its having been singled out by different writers as peculiar in one or another respect. When thus distinguished, qualities have been attributed to it more like those of the active leucocytes than those of the basophils of other species.

It has not been possible thus far to make any satisfactory study of the bone marrow under the benzidine-thionin stain, and the discussion of this vital phase of the question must be deferred until there may be found some method applicable to paraffin or other thin sections that will be more satisfactory than any available at present. Films of the fresh bone marrow are unsatisfactory for finer cytological study, since it has been impossible in such preparations to avoid a general sprinkling of the slide with free granules, and these adventitious bodies make a study of individual cells very difficult.

Another line of inquiry into which it has not been possible to go is that of comparative hematology. The field is large and considerable confusion appears to prevail in it, but it is possible that a study of the basophilic forms encountered at different levels of the evolutionary series might throw some light upon the present problem.

It is stated by Werzberg that the basophil does not appear in the teleost fishes except in the form (*Carassius auratus*) possessing the most highly developed blood picture. The eosinophil appears much lower in the scale. Once developed, however, the basophil appears to become relatively much more frequent than it is in the higher forms. Thus, Klieneberger and Carl find in the frog (*Rana esculenta*) 23 per cent of basophilic forms, 6 per cent of eosinophilic, and 26½ per cent of non-granular polymorphonuclear cells. Goodall found the basophils present "in considerable numbers." The occurrence of a high basophilic ratio is supported by the findings in a few preparations from material that has come to

hand incidentally during the course of the present study. The basophilic percentage in a tortoise (*Emys lutaria*) is quoted by Werzberg as from 6 to 8 per cent. The benzidine stain has shown a striking predominance of the basophilic forms in this animal, comparatively few of the granulocytes showing the brown benzidine reaction. The basophilic percentage obtained for the common fowl by various workers whose results are tabulated by Burnett varies from 1 to 4.3 per cent, but a much higher figure is indicated in films stained by the present method.

The apparent discrepancies in the various findings for the blood of the lower forms probably depend upon the marked water solubility of the basophilic granular substance in the blood concerned. There is a striking contrast in the appearance of films of chicken blood stained by the benzidine-thionin and by the usual eosinate methods. There is some indication that occasional cells in the blood of these lower forms may show a mixed granular reaction.

These fragmentary observations upon the blood of the lower animals serve to warn against too dogmatic a conclusion as to the interpretation to be placed upon the basophil. It is not to be questioned that a cell type present in any species in such predominating numbers as those quoted above may well be suspected of having a real functional significance, at least for the species concerned. Another consideration based upon the facts of comparative hematology is not to be lost sight of. While the number of basophils relative to the other white cells of the blood is uniformly small in mammals, it is fairly constant within each animal species. This may mean only that the leucocytes or marrow cells within the given species are subject to a fairly constant set of conditions leading to the appearance in them of a peculiar degenerative change, but, on the other hand, it may indicate that the basophil is not a dead or dying cell but a functioning blood unit possessing some obscure rôle in the general body economy. But while such a possibility is not to be disregarded, it is still believed that the evidence thus far at hand points in the main toward an interpretation of the cell as a pseudoleucocyte, and it is felt that the basophilic granule as found in mammals and particularly in man is not homologous with the neutrophilic and eosinophilic types and that the human basophil is not to be considered as a true leucocyte in the usual meaning of the term.

CONCLUSIONS.

1. The basophilic granule of blood and marrow cells does not show the brown color reaction with benzidine solutions that is characteristic of the neutrophilic and eosinophilic granules. It differs from these types also in other important microchemical and physical particulars.

2. The hemic basophil which bears these granules is peculiar from a purely cytological standpoint, while physiologically it appears to be devoid of any functional activity comparable with that of the other granulocytes.

3. The peculiarities exhibited seem in every instance, at least for mammalian blood, best explained as evidence that the basophil is a degenerated or degenerating cell. It is probably derived from the eosinophilic cells and perhaps in rare cases from those of neutrophilic type.

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EXPLANATION OF PLATE 15.

FIG. 1. Myelogenous leucemia. A field from a blood film stained with benzidine-thionin. *a*, eosinophilic myelocyte; *b*, neutrophilic myelocyte; *c*, polymorphonuclear neutrophil; *d*, basophil; *e*, early myelocyte; *f*, myelocyte with granules of mixed type.

FIGS. 2 to 9. Isolated cells from the blood of myelogenous leucemia and from a film of normal blood treated with an acid solution before staining. Benzidine-thionin method.

FIG. 2. Basophil of smaller type with heavily stained granules.

FIG. 3. Basophil of larger type with lobulation of the lightly stained nucleus and granules of the paler type. The granules are variable in size and shape.

FIG. 4. Eosinophilic myelocyte with a few gamma granules. Early nuclear changes.

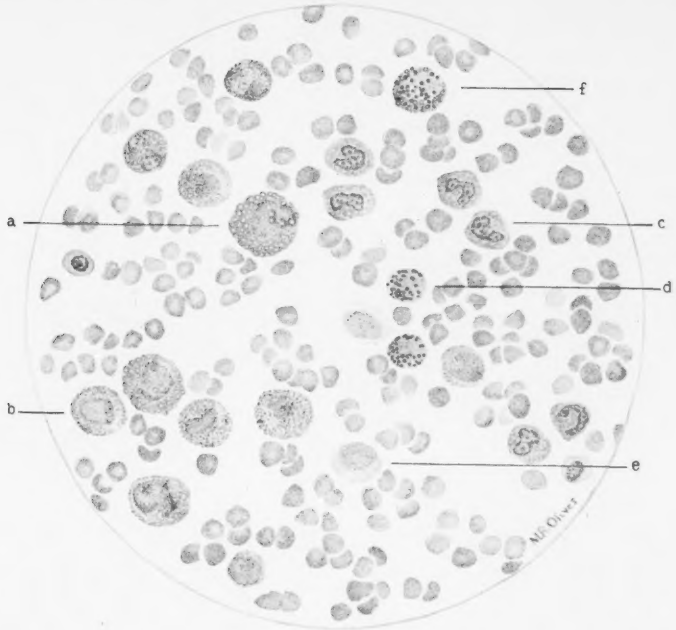
FIG. 5. Smaller cell with shrunken, more deeply stained nucleus. The gamma granules outnumber the eosinophilic.

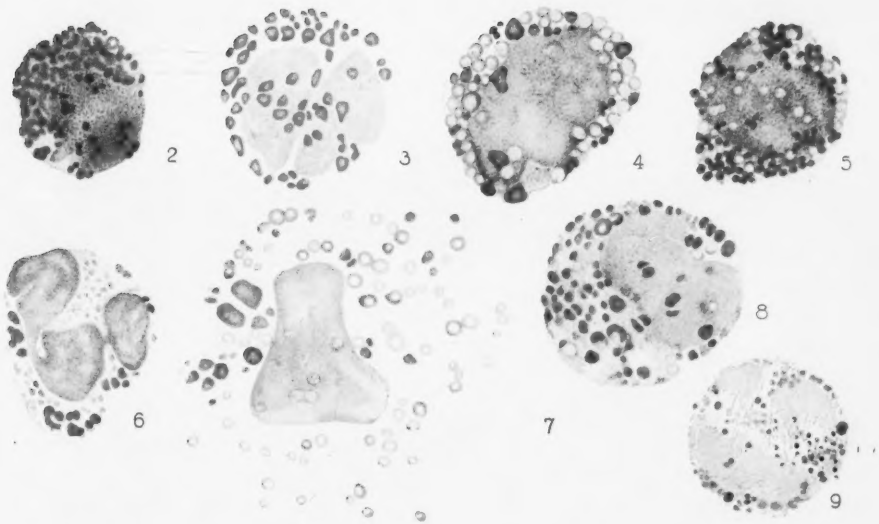
FIG. 6. Polymorphonuclear neutrophil with basophilic granular changes.

FIG. 7. Ruptured eosinophilic myelocyte. Basic staining material making its appearance in individual granules.

FIG. 8. Myelocyte with constricted nucleus and progressing granular changes.

FIG. 9. Polymorphonuclear neutrophil of normal blood with granular reaction altered by treatment with an acid solution before staining. Scattered granules exhibit a metachromatic thionin reaction.





(Graham: Hemic basophil.)